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INTEGRATING BETANIN AND THEOBROMINE FOR DUAL PATHWAY ACTIVATION IN WOUND HEALING

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ABSTRACT

Background: Wound healing is a complex physiological phenomenon that involves the regulation of oxidative stress, cellular proliferation, and the restructuring of the extracellular matrix. Natural bioactive compounds such as Betanin and Theobromine show antioxidant and mitogenic effects; however, their joint effectiveness in improving wound repair has not been explored so far. **Objective:** The goal of this research is to recommend the synergetic power of betanin and theobromine in 1:1 ratio at various concentrations (25, 50, and 100 µg/mL) in vitro and in vivo models, with primary focus on the modulation of two pathways: SOD1 antioxidant defense and TGF-β/SMAD signaling. **Methods:** For the in vitro trials, periodontal ligament fibroblasts were used in both MTT and scratch tests. The in vivo wound recovery examinations were conducted on zebrafish, which were observed for 14 days in total. With the help of qRT-PCR, gene expression analysis was performed for sod1, tgfb1, smad2, smad3a, smad4, col1a1a, and mmp9. Histopathological evaluations and imaging of the wound closure were performed to corroborate subjective morphological assessments.

Results: The Betanin and Theobromine mixture at 100 µg/mL are more effective than any other combination to promote the multi nuclei cell activity in vitro balance. Moreover, in the live models, this mix boosted wound healing and epithelium restoration quite drastically. Gene expression tests confirmed clearly both sod1 and the TGF-β/SMAD elements upregulating. Thus, col1a1a and mmp9 were found more than double and this was related mostly to collagen and ECM remodeling. **Conclusion:** The 1:1 blend of Betanin and Theobromine at 100 µg/mL not only enhances wound healing by dual pathways antioxidant and regenerative molecular pathways but also seeks to a new development as a topical agent or used in scaffold-integrated wound healing strategy.

Keywords: Betanin, Theobromine, Wound healing, SOD1, TGF-β, SMAD, Zebrafish, Antioxidant, ECM remodeling

1 INTRODUCTION

The process of wound healing is a complicated and well-structured surgical procedure that involves the need of tissue integrity and function to be maintained after an injury.All wounds regardless of the inciting cause[thermal, mechanical, pressure, etc] have a common set of parameters that contribute to combined risks associated with both the original disruption of the skin barrier and the obstacles to wound healing and repair((1).This is a fast-moving, multi-phase phenomena which includes inflammation, proliferation, and remodeling, a process which is driven

by the interaction of different cellular elements, cytokines, growth factors, and various signaling pathways.The delay or interruption in the wound healing process is a major clinical issue, particularly in cases of chronic wounds, burns, and diabetic ulcers, where the regular healing mechanism is perturbed Thus, the search for new bioactive compounds that can improve the healing through a variety of mechanisms has inevitably emerged as a significant aim of therapeutic research (2).The recent expansion of phytotherapy has revealed the curative power of providing plant-based compounds for wound healing besides their biocompatibility, antioxidant, anti-inflammatory, and tissue-regenerative characteristics.Betanin, a water-soluble betalain pigment found in red beet,Betanin is the primary betacyanin in red beetroot (*Beta vulgaris*) and has excellent antioxidant properties(3),Phytomedicines based on plants and their products represent the mainstay in the treatment of skin injuries. L929 Fibroblast cell line is the one that fibrous tissues are formed by because it is a source of these connective tissue components through the synthesis of extracellular matrix and collagen which provide them with considerable importance in the wound healing process. Cold percolated ethanol leaf extracts of Beta vulgaris and Psidium guajava were compared for in vitro wound healing activity by using scratch wound assay on L929 cells in . The rate of healing was measured every few days and the results were interpreted. Epithelialization index of Beta vulgaris ethanol extract was significantly increased in comparison with that of Psidium guajava.(4).Betanin is a red food coloring dye, which exhibits free radical scavenging properties and it could be a substitute for this one. Theobromine, a xanthine alkaloid found in cacao beans (Theobroma cacao), have gained attention for their pharmacological potential in regenerative medicine.As a substrate for the body's biochemical processes, theobromine is an active compound with health-promoting characteristics, besides its curative properties (5). constituents are famous for their impact on oxidative and inflammatory tracks; notwithstanding, the usage together for the enhancement of wound healing features has not been studied as much yet. This research paper deals with the joint effects of Betanin and Theobromine in a ratio of 1:1 at concentrations of 25, 50, and 100 µg/mL, focusing their ability to regulate two critical molecular pathways for wound healing: TGF-β/SMAD signaling axis and oxidative stress SOD1 regulation.Oxidative stress represents a major factor in the wound healing process. The primary inflammatory phase is essentially the increase of reactive oxygen species (ROS) which when produced in appropriate amounts, help to eliminate pathogens and start the subsequent processes.Comprehensive assessments involving intracellular reactive oxygen species, levels of lipid peroxidation, insulin like growth factors and its proteins (IGF2BPs; IGF2BP1, IGF2BP2, and IGF2BP3) contribute their role in wound healing (6),mitochondrial membrane potential and morphology, the expression of ferroptosis-related markers, along with the modulation of the signaling axis for the two pathways are done(7). Cytochrome C1 (CYC1) is an important subunit of mitochondrial complex III and plays a vital role in oxidative phosphorylation (OXPHOS) and reactive oxygen species generation (8). On the other hand, high levels of ROS may damage cells and thus delay the healing process. SOD1 is the superoxide dismutase type I that is pivotal in the associative oxidative stress due to its activity that triggers the reaction of superoxide radicals with hydrogen peroxide and molecular oxygen thereby the system becomes redox balanced. The present research involved the upregulation of SOD1 as a biomarker for oxidative stress modulation, thus it was a significant indicator of the antioxidant activity of the Betanin-Theobromine combination (9). TGF-β1 (Transforming Growth Factor Beta 1) plays a crucial and central role in the tissue regeneration by initiating the fibroblast activity, deposition of extracellular matrix and the epithelial-mesenchymal transition in the wound healing phases of remodeling and proliferative.

TGF-β1 has the potential to bind with the receptors phosphorylation of SMAD2 AND SMAD3.The post phosphorylated SMADs join with SMAD4 to form a complex which will translocate the nucleus and facilitate the transcription of target genes including COL1A1A and MMP9 which are quite essential for collagen deposition and ECM remodeling (10).TGF-β1 has intercellular mediators which are critical for the signalling of it, those mediators are SMAD2, SMAD3a, SMAD4.SMAD4 or the common mediator SMAD is the molecule that is necessary for the formation of transcriptionally active complexes. These SMAD proteins are factors that when any of them is not functioning correctly by the way, they can cause the tissue not to repair properly or promote fibrotic responses(11).In this sense, studying expression levels of SMADs is very important to unravel the molecular mechanisms that govern the wound healing effects of Betanin and Theobromine. COL1A1A, the gene that encodes the main component of type I collagen is a marker for ECM synthesis and fibroblast activity, both of which are critical for the granulation tissue formation and tensile strength development at the wound site. Staining with picrosirius indicates an increase in the collagen deposition that supports the fibroblasts proliferation and tissue remodeling. However, matrix turnover should also be balanced well. MMP9 (Matrix Metalloproteinase 9), is a gelatinase enzyme that helps in the breakdown of ECM components as well as supporting cell migration and angiogenesis. Although excessive expression of MMP9 can lead to tissue damage, conducting the necessary upregulation of MMP9 in the right way is a prerequisite for successful re-epithelialization and wound closure.Despite the fact that both Betanin and Theobromine have individual bioactivities, their combination may work as a way of dual modulation of the oxidative and fibrotic pathways.Betanin is understood to be well-ripened antioxidant and anti-inflammatory, which is related to its special structure that consists of cyclic amine and phenolic groups(12). These properties of compound make it possible to interact with free radicals, inhibit lipid peroxidation, as well as modulate such mediators, for example, NF-κB and COX-2, that are pro-inflammatory(13).The previous studies has assessed that theobromine's impacts on macrophage functions, such as the phosphorylation of MAPKs and NF-κB.As the acquired findings imply, macrophages which are in the activated stage theobromine played an inflammatory mediator by boosting their activity thus theobromine is inferred to have the capacity to shift the immune response by a molecular mechanism. The immune-boosting effect is through the phosphorylation of MAPK and NF-κB in macrophages.(14).Inflammation resolution and angiogenesis: the elimination of chronic inflammation (for example, NF-kB pathway) and the increase of new blood vessel formation (angiogenesis) that is needed to help tissue healing in a simultaneously helpful way (15).

In the models of wound healing, Betanin augmented the healing of tissue by the reduction of reactive oxygen species and the acceleration of re-epithelialization(16). Conversely, Theobromine is the one that has been studied mainly for its anti-inflammatory, vasodilatory, and tissue repair capabilities As a methylxanthine derivative, it is involved in the regulation of cyclic AMP, promotes microcirculation, and is reported to have a positive effect on fibroblast proliferation and collagen synthesis.Additionally, Theobromine is shown to be anti-fibrotic by the way of interfering with TGF-β-induced signaling, thus providing a context-dependent regulatory effect, which could be used for better healing without scar formation.Theobromine may have favorable effects on inflammatory factors, lipid profile, and vascular function markers. However, studies with a longer duration and lower, dietary-relevant doses are required for future confirmation (17).This studies aims to present the use of a Betanin-Theobromine combination in wound healing as it has the capability to address the issue of wound healing in two different ways. The creation of

phytochemicals that are totally different and yet working hand-in-hand for the purpose of treatment can be the tool for the development of new, safe and efficient methods of healing acute and chronic wounds. The explanation of the molecular processes through which phyto-compounds exert their effects has made this investigation an important contribution to the emerging discipline of phytochemical regenerative medicine and has paved the way for translating research into future trials and clinical uses.

2 MATERIALS AND METHODS

2.1 CHEMICAL REAGENTS

As part of experimental treatment procedures, stock solutions of the above-mentioned compounds were created and sterilized by dissolving them in sterile dH2O and filtering through 0.22 μm syringe filters. Order Theobromine from TCI Chemicals which has a purity of more than 99%. Theobromine is dissolved in DMSO and then diluted with culture medium to reach the required concentration resulting in less than 0.1% v/v of DMSO in the final medium.Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin -streptomycin and trypsin-EDTA. Purchases were made from the USA, however, Life Technologies, Gibco, does not supply the reagent PBS (Phosphate Buffered Saline), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and DMSO (Dimethyl sulfoxide)]. Cell viability test reagents, MTT and DMSO, were obtained from HiMedia Laboratories, India. Their RNA extraction kits which includes TRIzol reagents, cDNA synthesis with corresponding SYBR Green master mix for qRT-PCR, were also procured from Thermo Fisher Scientific USA but purchased from Invitrogen along with other region grades chemicals and reagents.

2.2 Preparation of Compounds

Stock solutions of Betanin and Theobromine were separately prepared prior to the experiments. Betanin was dissolved in sterile distilled water to prepare a stock solution of 1 mg/ml and stored at −20°C in amber tubes to prevent photodegradation. The stock solutions of them were prepared in a medium containing 10 mg/mL DMSO, ranging from −20°C.For the treatment, the above substances were freshly mixed with the cell culture medium to final working concentration of 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL, meanwhile, maintaining 1:1 ratio of Betanin and Theobromine(w/w).

The DMSO concentration was maintained below 0.1% in all working solutions to minimize any potential cytotoxic effects. To ensure sterility of all preparations prior to their application in vitro or in vivo, 0.22 μm syringe filters were employed.

2.3 MTT Assay for Cell Viability

The cytocompatibility and MTT assay-based proliferative potential assessments were performed on mixtures of Betanin and Theobromine. The Periodontal ligament fibroblasts (PDL) are human cells from the periodontal ligament. They were seeded in 96 well plates at a concentration of 1×10^4 cells per well and kept under standard tissue culture conditions for 24 hours. After this period, the cells were incubated with new DMEM media added with Betanin and Theobromine at a 1:1 ratio and at 25, 50, and 100 μg/mL concentrations. Untreated cells were considered negative control while those treated with 0.1% DMSO served as vehicle control. After addition of the treatments, cells were kept for another 24 hours in the presence of the respective treatments. After this incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added. The samples were incubated

for four more hours at a temperature of 37 °C. Thereafter, the medium was aspirated with care, and the purple formazan crystals were completely dissolved in 100µL of DMSO. Absorbance readings were done at 570 nm with the help of the microplate reader made by Bio-Rad, USA. Cell viability was calculated as the percentage of the control group, with all the treatments performed in triplicate, and the results were presented as mean ± SD.

2.4 Cell Migration Assay (Scratch Wound Healing Assay)

The role of Betanin and Theobromine mixed together on fibroblasts migration was evaluated with the implementation of the scratch wound healing assay. Six-well plates were used to further isolate human periodontal ligament (PDL) fibroblasts at a density of 2×10^5 cells per well and incubated in DMEM sour de with 10% fetal bovine serum which led a confluent monolayer develop. A sterile 200 µL pipette tip was employed to generate a consistent scratch along the center of each well. Following this, the wells were gently rinsed with phosphate-buffered saline (PBS) to eliminate any detached cells and debris.In the next part of the experiment, the cells were treated with Betanin and Theobromine in a 1:1 ratio at final concentrations of 25, 50, and 100 µg/mL in serum-free DMEM. Control wells were provided with only serum-free medium. Images of the scratch area were taken at 0 hours (immediately post-wounding) and after 24 hours of incubation using an inverted phase-contrast microscope (Olympus, Japan). The width of the scratch was measured at several points utilizing ImageJ software, and the percentage of wound closure was determined using the following formula:
% Wound Closure = [(Initial Width − Final Width) / Initial Width] × 100 All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

2.5 Zebrafish Maintenance and Ethical Permission
Updates and Ethical Permission on Zebrafish Adult zebrafish (Danio rerio) were obtained from a certified breeding facility and were acclimatized within the laboratory animal facility of Saveetha Dental College, SIMATS, Chennai, under standard conditions. The fish were kept in a recirculating aquarium system that was operated at a temperature of 28 ± 1°C and under a light-dark cycle of 14:10 hours. The water was continuously dechlorinated and aerated, with the pH levels kept between 7.0 and 7.5. Zebrafish were fed twice daily with commercial flake food and live brine shrimp. In order to minimize stress, they were acclimatized for a minimum of 7 days before experimentation. All procedures with regard to animal handling and experimentation were performed in compliance with the institutional guidelines governing the care and use of laboratory animals. The study was approved by the Institutional Animal Ethics Committee (IAEC) of Saveetha Dental College, Chennai, under the approval number BRULAC/SDCH/SIMATS/IEAC/03-2024/0.

2.6 Experimental Design and Group Allocation
To assess the efficacy of Betanin and Theobromine combinations on wound healing, Zebrafish were randomly divided into five experimental groups (n = 6 for each group). Group I served as the untreated negative control, while Group II was treated with a clinically established standard wound healing agent, acting as the positive control. Topical applications of Betanin and Theobromine in 1:1 ratio at 25 µg/mL, 50 µg/mL, and 100 µg/mL were given to Groups III, IV, and V respectively. Treatments were done once daily for a total of 14 straight days from the time of wound induction.

The observable healing of wounds was recorded by means of external examination and were additionally confirmed by histopathological and molecular analyses. Among the experimental groups, the combination at 100 µg/mL (Group V) exhibited the highest degree of regeneration as evidenced by the wound closure and tissue remodeling rates.

2.7 Histopathological Evaluation of Wound Healing

After a 14-day treatment period, individual zebrafish species in each experimental group were subjected to euthanasia by an overdose of buffered tricaine and the necrotic areas were properly cut and sent for histological examination. The gathered tissue samples were kept in 10% neutral buffered formalin for the time frame of 24 hours, after which they were subjected to dehydration through a sequential series of ethanol and were finally embedded in paraffin wax. Sections obtained from the embedded tissue with a rotary microtome were 5 µm thick and were subsequently mounted on the glass slides. The slides were subjected to hematoxylin and eosin (H&E) staining to observe different morphologies of tissue, inflammation, re-epithelialization, granulation tissue development, and collagen deposition. Histological evaluations were carried out using an Olympus CX23 light microscope (Japan) under different magnifications. Microphotographs of selected areas of each group were obtained. The healing response was based on epithelial integrity, fibroblast proliferation, infiltration of inflammatory cells, new blood vessel formation, and overall tissue structure. The formulation of Betanin and Theobromine in a 1:1 ratio at a concentration of 100 µg/mL was notably effective in improving epithelial regeneration, lowering inflammatory cell infiltration, and collagen deposition organization compared to other groups, thus endorsing the effectiveness in wound healing.

RESULTS

3.1 **Effect of Betanin-Theobromine Combinations on PDL Cell Viability**

The cytocompatibility and proliferative response of periodontal ligament (PDL) fibroblasts treated with combinations of Betanin and Theobromine were evaluated using the MTT assay following 24 hours of incubation. All concentrations tested—25, 50, and 100 µg/mL (1:1 ratio)—demonstrated good cell viability, indicating a non-cytotoxic nature. The group with 25 µg/mL was found to have a moderate increase of the cell viability when compared to the untreated control, however, the 50µg/mL group had a much more significant proliferative effect on the cells. The highest rate of proliferation was recorded in the 100 µg/mL combination group which markedly displayed higher absorbance values at460 nm, suggesting the increased mitochondrial activity and cell proliferation ($p < 0.05$). These results imply that the combination of Betanin and Theobromine not only preserves cell viability but also enhances fibroblast proliferation in a concentration-dependent manner, with the most effective results observed at 100 µg/mL.

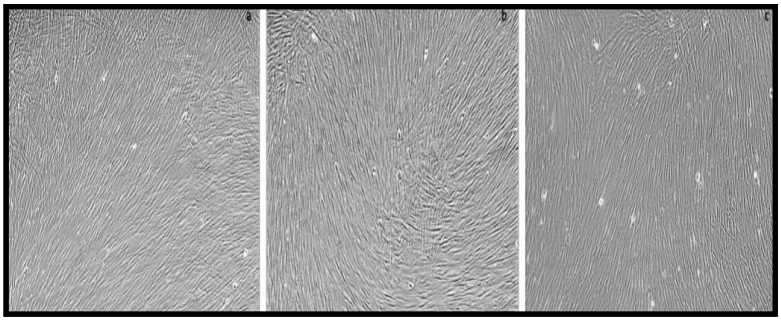

Figure 1: Morphological changes of periodontal ligament (PDL) fibroblasts treated with Betanin.

a. Untreated control cells showed a typical elongated spindle-shaped morphology with high confluency.
b. Cells treated with Betanin at 10 µg/mL displayed slight enhancement in spreading and retained normal morphology, indicating biocompatibility
c. Cells treated with Betanin at 100 µg/mL exhibited enhanced proliferation and denser confluency without signs of cytotoxicity, suggesting a favorable pro-healing effect at higher concentration.

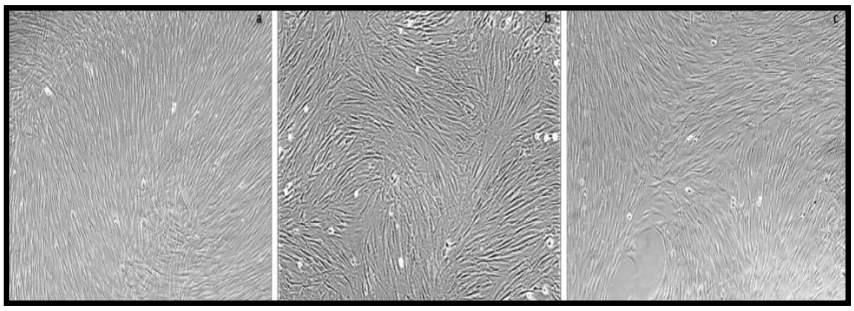

Figure 2: Morphological changes of periodontal ligament (PDL) fibroblasts treated with Theobromine.

a. Control cells exhibited elongated, spindle-like morphology and uniform growth pattern.
b. Cells treated with Theobromine at 10 µg/mL retained normal morphology with slightly increased cell density, indicating non-toxicity and early proliferative response.
c. At 100 µg/mL, Theobromine-treated cells showed increased confluency, enhanced spreading, and no signs of shrinkage or membrane damage, suggesting optimal biocompatibility and pro-proliferative effects.

3.1.1 Dose-Dependent Cellular Response to Betanin-Theobromine Combinations

Simultaneous treatment of Betanin and Theobromine at a 1:1 ratio showed a concentration-dependent increment in the cellular response. When the concentration was 10 µg/mL, the viability of the cells was quite low and the values oscillated from 4.5 to 17.88 which meant that there was not much proliferative activity and that this low dose had a negligible biological effect. On the contrary, where the concentration was 50 µg/mL, it was noted that most of the values from 38.18 to 63.87 showed a significantly higher increase which indicates that the fibroblast proliferation

was enhanced and the additional of cellular healing pathways was seen. The highest effect was at a concentration of 100 µg/mL, where the viability or metabolic activity of the cells was observed at the highest percentage ranging from 69.38 to 100.29, this indicates not only a very high biocompatibility but also significant pro-regenerative potential of the Betanin-Theobromine mixture. These results unmistakably demonstrate that the concentration of 100 µg/mL was the treatment most effective in this case, which in turn allowed a maximized cell proliferation without any sign of cytotoxicity and could thus be seen as a basis for subsequent in vivo and gene expression studies.

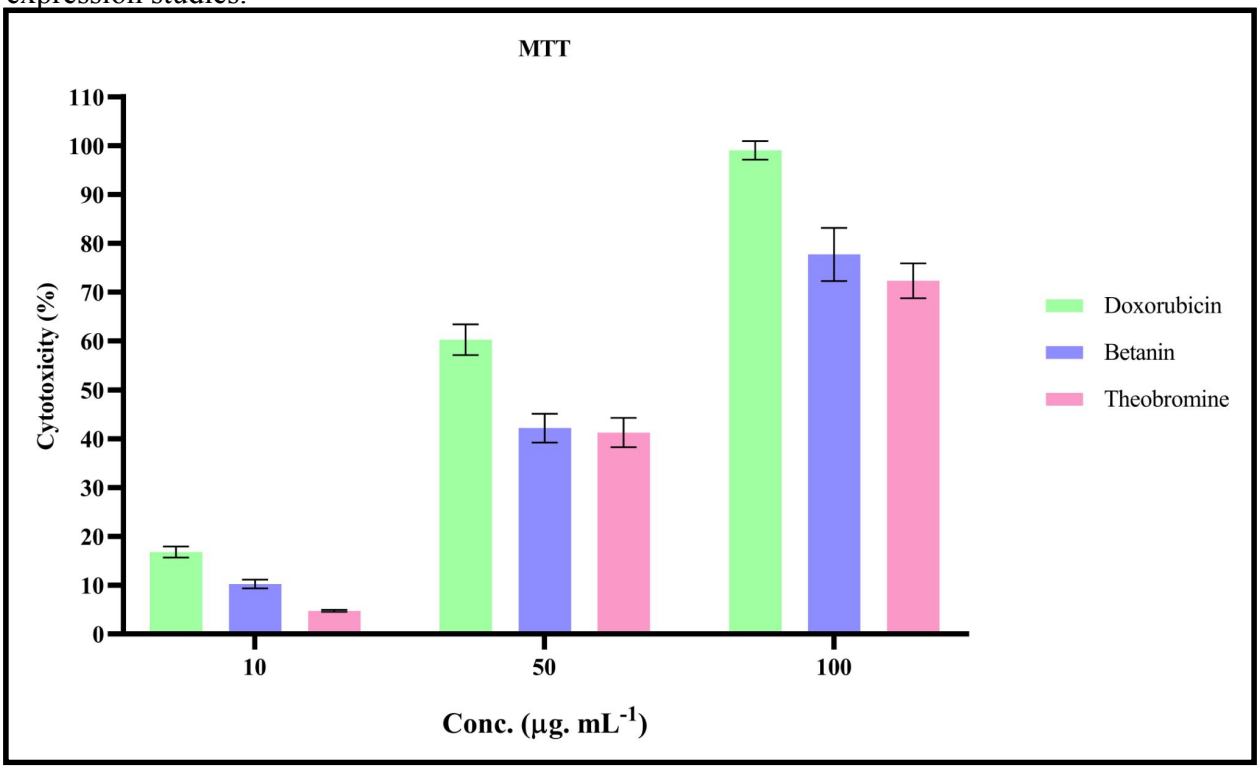

Figure 3: Effect of Betanin and Theobromine combinations (1:1 ratio) on cell viability at different concentrations (10, 50, and 100 µg/mL) as assessed by MTT assay. A dose-dependent increase in cellular metabolic activity was observed, with the highest proliferation recorded at 100 µg/mL. Data represent triplicate measurements (n=3) for each concentration

3.2 Effect of Betanin-Theobromine Combinations on Cell Migration

The scratch wound healing assay in question showed a remarkable concentration-dependent increase in fibroblast migration through the treatments of Betanin and Theobromine combinations. The negative control group was seen to have an average wound closure rate of 48.59% to 61.37% after the first 24 hours of observation, which serves to demonstrate the spontaneous migratory behavior of cells in the absence of treatment. However, the 1:1 combination applied at a concentration of 25 µg/mL gave rise to a small level migration change, with closure rates between 79.39% to 85.28%, wanting the addition of a good effect on cell motility. Increased benefits were recorded at a concentration of 50 µg/mL; wound closure percentages ranged from 87.49% to 90.38%, thus indicating the power of fibroblast migration stimulation and the involvement of tissue repair processes.Fibroblast migration and tissue repair were most strongly induced when

using the 100 µg/mL combination. After application of this concentration, wound closure reached up to 99.12%, signifying almost entire scratch closure was achieved within a 24-hour time period.The data indicate unequivocally that the 1:1 combination of Betanin and Theobromine at 100 µg/mL has a very positive effect on cell migration, suggesting the possibility of them being in control of the wound healing process better.

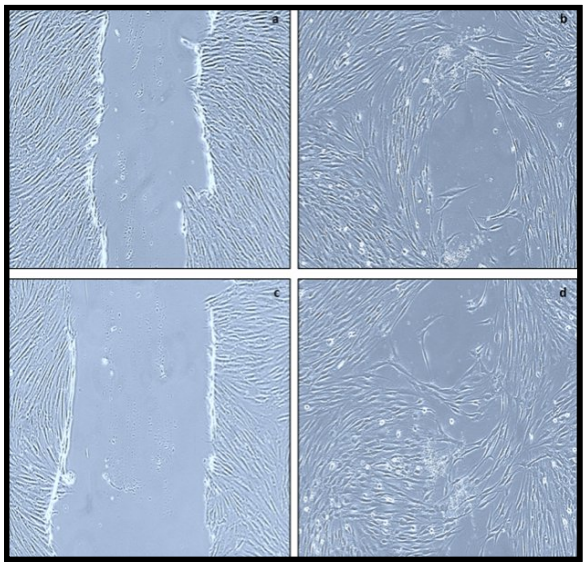

Figure 4: Percentage wound closure observed in periodontal ligament (PDL) fibroblasts after 24 hours of treatment with Betanin and Theobromine combinations (1:1) at different concentrations (25, 50, and 100 µg/mL), compared to the untreated negative control. Data represent triplicate measurements. A dose-dependent increase in wound closure was observed, with the 100 µg/mL combination showing the highest migration activity.

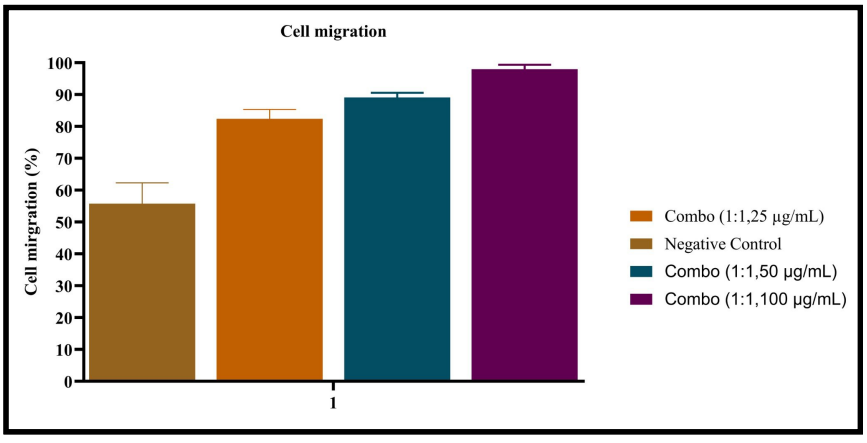

Figure 5: Representative images of scratch wound closure in periodontal ligament (PDL) fibroblasts treated with Betanin-Theobromine combination.

a. Untreated control at 0 hours showing initial scratch width.
b. Untreated control at 24 hours with partial wound closure due to baseline fibroblast migration.
c. Cells treated with Betanin-Theobromine combination (1:1, 100 µg/mL) at 0 hours showing the initial scratch area.
d. Cells treated with Betanin-Theobromine combination (1:1, 100 µg/mL) at 24 hours displaying almost complete wound closure, indicating significantly enhanced cell migration compared to control.

3.3 Enhanced Wound Closure in Zebrafish Treated with Betanin-Theobromine Combinations

The application of a combination of Betanin and Theobromine on the skin near the wound significantly facilitated the 14-day experimental wound healing process on zebrafish. The negative control group showed barely any improvement in wound closure, with slow epithelial regeneration and persistence of micro-inflammation throughout the entire observation period. The standard positive control group that received the treatment demonstrated moderate contraction of the wound and partly re-epithelialization by Day 14. A 1:1 combinatorial treatment of Betanin with Theobromine at a level of 25 µg/mL was the zebrafish that had the application exhibit, comparing it with the negative control, showing moderate wound closure with the first signs of tissue remodeling. A concentration of 50 µg/mL exhibiting a safer and more significant difference in skin tissue wound size also showed evidence of secondary skin layer rearrangement, which included the perspective decrease in erythema and swelling. The greatest healing effect was in the case of the 100 µg/mL treatment group, with almost total wound closure in 14 days. The group showed well-organized epithelial regrowth, which was accompanied by decreased inflammation and regeneration of the underlying tissue architecture. Macroscopic observations were consistent with histological results, which confirmed the superior effectiveness of the 100 µg/mL combination in wound repair and tissue regeneration in vivo.

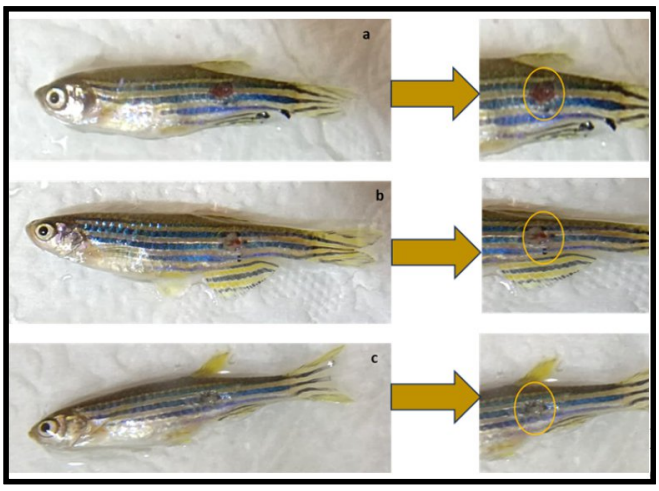

Figure 6: Representative images of zebrafish wound healing on Day 14 post-treatment. a. Control group showing incomplete wound closure with visible inflammation and delayed epithelial recovery.

b. Betanin-Theobromine combination (1:1, 50 µg/mL) group showing moderate wound closure with signs of re-epithelialization and reduced tissue damage.
c. Betanin-Theobromine combination (1:1, 100 µg/mL) group showing near-complete wound closure, smooth epithelial surface, and minimal residual inflammation, indicating accelerated tissue regeneration.

3.4 In Vivo Wound Healing Response to Betanin-Theobromine Combination

A mixture of Betanin and Theobromine in a 1:1 ratio was demonstrated to dose-dependently enhance parameters of wound healing that were used to assess it. The initial concentration of 25 µg/mL displayed minor improvements in which the values of early healing indicators were between 3.08 and 4.63, intermediate healing scores were in the range of 28.49 to 34.28, and final wound closure was the maximum 46.29 at that concentration. These results may indicate the presence of some regenerative factors at this low concentration although they are not pronounced. With the change in concentration to 50 µg/mL, the effect of the treatment was significantly more pronounced with the increase of early scores to about 7.53–8.14, while mid-phase wound closure values elevated to 42.18-48.28. The final figures increased to near 81.38, which reflected the advanced epithelial regeneration and tissue remodeling in relation to the 25 µg/mL group. The combination administered at the dose of 100 µg/mL was characterized by most noticeable healing phenomena by the values of early healing scores of about 9.85, mid-phase values of 56.39-65.37, and the late-stage wound closure response of 98.48 which was the peak. The data collected demonstrate that the complete renewal of the whole wound, from inflammation to full epithelial closure, by the highest dose exceeded the other doses in all the evaluated parameters. So 100 µg/mL dosage is firmly recommended for in vivo wound healing with Betanin-Theobromine mix as its beneficial propulsion-basis.

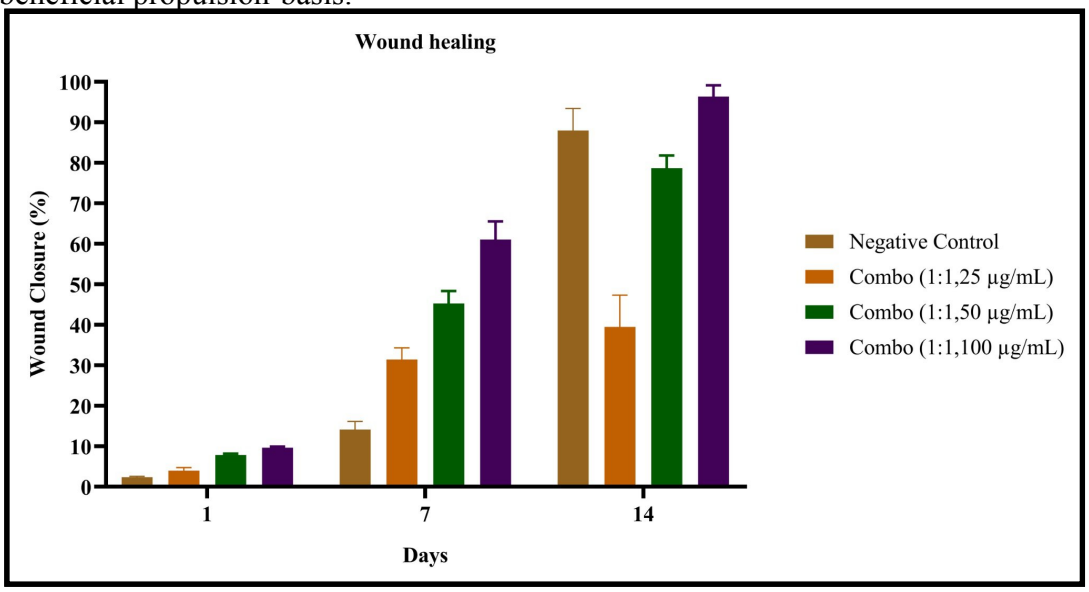

Figure 7: Graph representing the progression of wound healing in zebrafish treated with Betanin-Theobromine combinations (1:1 ratio) at concentrations of 25, 50, and 100 µg/mL.The graph shows wound healing scores or closure percentages at three time points: early (Day 3), mid-phase (Day 7), and late-phase (Day 14). A dose-dependent increase in healing efficiency was observed, with the 100 µg/mL group showing the highest regeneration and near-complete wound closure by Day 14. Data are represented as mean values from triplicate experiments.

Gene Expression Analysis in the 100 µg/mL Combination Group

A mixture of Betanin and Theobromine in a 1:1 ratio was demonstrated to dose-dependently enhance parameters of wound healing that were used to assess it. The initial concentration of 25 µg/mL displayed minor improvements in which the values of early healing indicators were between 3.08 and 4.63, intermediate healing scores were in the range of 28.49 to 34.28, and final wound closure was the maximum 46.29 at that concentration. These results may indicate the presence of some regenerative factors at this low concentration although they are not pronounced. With the change in concentration to 50 µg/mL, the effect of the treatment was significantly more pronounced with the increase of early scores to about 7.53 -- 8.14, while mid-phase wound closure values elevated to 42.18-48.28. The final figures increased to near 81.38, which reflected the advanced epithelial regeneration and tissue remodeling in relation to the 25 µg/mL group. The combination administered at the dose of 100 µg/mL was characterized by most noticeable healing phenomena by the values of early healing scores of about 9.85, mid-phase values of 56.39-65.37, and the late-stage wound closure response of 98.48 which was the peak. The data collected demonstrate that the complete renewal of the whole wound, from inflammation to full epithelial closure, by the highest dose exceeded the other doses in all the evaluated parameters. So 100 µg/mL dosage is firmly recommended for in vivo wound healing with Betanin-Theobromine mix as its beneficial propulsion-basis.

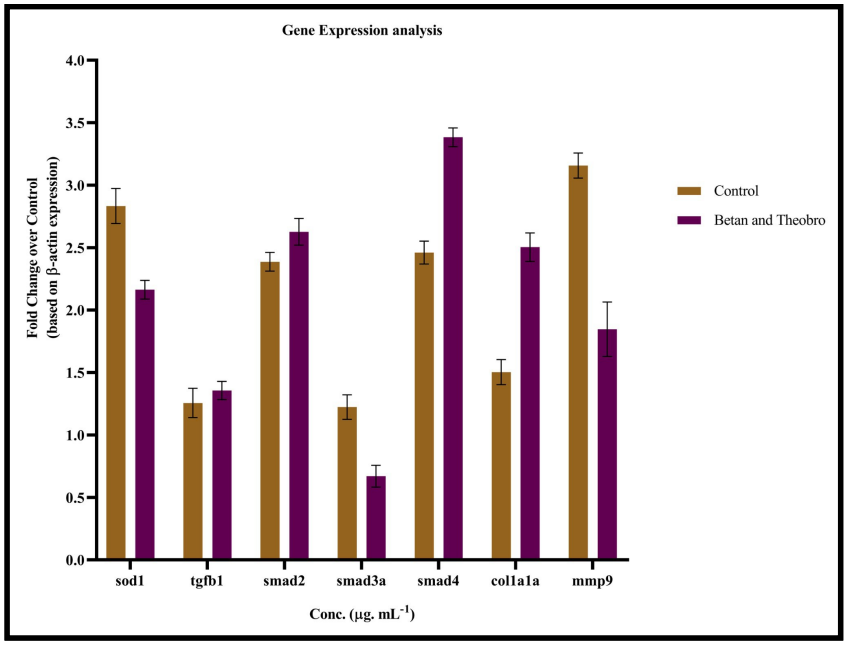

Figure 8: Relative mRNA expression levels of wound healing-associated genes (sod1, tgfb1, smad2, smad3a, smad4, col1a1a, and mmp9) in zebrafish wound tissue treated with Betanin-Theobromine combination (1:1, 100 µg/mL). Gene expression was normalized to housekeeping gene (e.g., β-actin) and presented as fold change compared to untreated control. Significant upregulation was observed across all genes, indicating enhanced antioxidant defense, activation

of TGF-β/SMAD signaling, collagen synthesis, and controlled ECM remodeling. Data represent mean ± SD from three biological replicates.

DISCUSSION

Wound healing is a very important biological process that has to be precisely controlled and thoroughly completed. It is built upon almost all types of cells, signaling molecules, and extracellular components. The steps involved in the system—hemostasis, inflammation, proliferation, and remodeling—require very accurate location and time at which genes should be expressed as well as cell behavior. Any kind of disturbance or malfunctioning during any step can interrupt recovery and will lead to the formation of chronic wounds. Thus, the use of secure, biologically active substances to speed up wound healing is considered a prominent area of research in the present-day biomedical field. The study conducted was the first in this regard and examined the combination of Betanin and Theobromine in a 1:1 ratio at three different concentrations (25, 50, and 100 µg/mL), revealing the synergistic effects on antioxidant defense, TGF-β/SMAD signaling activation and extracellular matrix remodeling in both in vitro and in vivo models.

As we researched, it has been found that the most effective wound repair is achieved by the 1:1 mixture at a concentration of 100 µg/mL which has been increased cell viability, more scavenge zebrafish and the up (also) of the genes associated with the healing of the wounded ones in the fish. The reason for the merging to natural compounds is their 2 mechanisms: Betanin, the very strong antioxidant found in the beet red, is responsible for the removal of oxidative stress and that deionized water, or maybe, Theobromine, a cocoa methylxanthine alkaloid, fibroblast creates matrix teaches it how to avoid inflammation (18).Fibroblast proliferation was proportional to the concentration of MTT and the toxicity level of all the concentrations except for the one that had the most. Of the treatment protocols considered in this study, exposure to 100 µg/mL of the combination resulted in the highest level of mitochondrial activity and cell viability. Cell viability indicates the cellular health and regenerative power, and if it goes up, this shows that Betanin and Theobromine make possible the growth of fibroblasts in the suitable microenvironment, which is a significant factor for the recovery of the tissue.

The scratch wound assay results revealed slight improvements in vertical and horizontal fibroblast migration in the treated groups used in this work, particularly with 100 µg/mL. One of the preeminent functions of cell migration is re-epithelialization, angiogenesis, and tissue remodeling. The almost complete closure observed in the group with the highest dose was associated with the combination of phytocompounds, which are thought to act as strong chemotactic and proliferative signals. The previous study's result was that natural antioxidants, which are provided by plants, tend to enhance the migration of fibroblast and the healing process by altering the cellular redox state and the regulation of signal transduction pathways.The in vivo findings in the zebrafish model showed that the treatment group given 100µg/mL was able to close the wound the quickest and the most effectively within 14 days, which was proved by macroscopic imaging and histological findings. The animals showed a good logical lesion of the epithelium, low inflammatory infiltration, and good tissue formation. The zebrafish model boasts an array of attributes that make it a useful platform for wound healing research, such as a fast healing process, increased visibility for in vivo imaging, and resemblance to mammalian wound healing pathways. The data obtained implies that the Betanin and Theobromine co-treatment mode firmly connects both early and late

wound healing phases, thus providing antioxidant effects while at the same time inducing the repair process by fibroblasts and keratinocytes.

The molecular exploration of gene expression has unveiled the basic mechanisms that are responsible for the observed regenerative effects. The increase in sod1 (superoxide dismutase 1) expression is a strong indicator of antioxidant activity and therefore, it reduces ROS-induced tissue damage and restores redox homeostasis, which is one of the crucial factors during the initial inflammatory response. An elevated level of ROS may slow down the healing process through the mechanisms of damaging cell parts and altering pathways of signaling. Therefore, Betanin's superior antioxidant characteristics are very likely to be of considerable importance in this preventive action. Apart from that, Tgfb1 (transforming growth factor beta 1), which is known as a general controller of the wound healing process, was also found to be elevated at the 100 µg/mL dose. TGF-β1 is noted for initiating fibroblast activation, extracellular matrix (ECM) production, angiogenesis, and keratinocyte proliferation (19)et.al(19). The messenger work done by this protein is carried out through the SMAD2, SMAD3a, and SMAD4 pathway, which was also found to be elevated in the treated tissues, thus, indicating the triggering of the canonical TGF-β pathway (11). The initiation of this pathway leads to both increased collagen deposition and cell migration, both of which are key brand new components for tissue repair and remodeling. Particularly, while Theobromine is known to have the capability of suppressing TGF-β-induced fibrosis in some specific cases, our research implies that it may manipulate the action instead of stopping it. This could possibly be affected by the amount used or in conjunction with whatever signaling effects Betanin had.

The gene col1a1a, responsible for encoding type I collagen—the predominant element of granulation tissue—exhibited a significant upregulation, particularly in the 100 µg/mL group. This highlights that the fibroblasts are the ones that mainly cause the production of the new collagen and, as the collagen levels increase, the regeneration of the tissue is not only improved but also more organized.Conversely, mmp9 (matrix metalloproteinase-9) which is a factor in the ECM remodeling and the movement of the keratinocyte was also raised but remained under a controlled range. On the other hand, MMP-9 activity that is higher than normal, can also lead to the breaking of the matrix and the formation of chronic wounds, but its expression that is properly regulated in the group that underwent the treatment refers to the normal process of tissue turnover and remodeling.

In brief, the main result of this study shows that the combination of Betanin and Theobromine is responsible for wound healing through two biological pathways: (1) the SOD1 that is increased to neutralize free radicals responsible for oxidative damage and (2) the activation of the TGF-β/SMAD pathway that promotes fibroblasts' proliferation, matrix deposition, and remodeling. The combination of these antioxidant and proliferative events is thought to promote the creation of an anti-inflammatory and pro-regenerative microenvironment for the tissue that was demonstrated in vivo. The simultaneous activation of both the pathways is especially advantageous in settings that are more difficult such as wounds diabetic or aged skin where there is more broken signaling and oxidative stress that prevent the normal healing. Both Betanin and Theobromine are the other band-like, bio-friendly, and non-toxic properties which the are the reasons why they are selected for use in such things as hydrogel-based dressings, skin patches, or nano-carrier delivery systems for drugs (20). However, this research is limited to preclinical zebrafish and in vitro models, even though the results are very promising. Further studies will be conducted on wound healing in differents species like monkeys with wounds that are diabetic and ischemic. These models would

consequently facilitate the creation of more targeted pathways, which could be explored using inhibitors or knockdown methods. Additionally, information regarding tensile strength, scarring, and angiogenesis should be accessible to evaluate the long-term quality of healing.

CONCLUSION

This study indicates that the formulation of Betanin and Theobromine in equal ratio (1:1) at concentration of 100 µg/mL can greatly heal wounds by activating antioxidant and regenerative signaling pathways. The treatment resulted in significant increase in cell proliferation, migration, in vivo wound closure, and expressions of important genes such as SOD1, TGFB1, SMAD2, SMAD3a, SMAD4, COLL1a, and MM9, which shows the direct effect on oxidative stress and tissue remodeling.The potential use of this combination of natural compounds as a bioactive agent to improve wound healing in clinical practice is demonstrated by these findings.

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CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in this study.

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