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Homeopathy and Photobiomodulation for Healing Diabetic Wounds *in vitro*

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Abstract

Photobiomodulation (PBM), as well as plant extracts of *Calendula officinalis (Calen)*, *Hypericum perforatum (Hyper),* and *Echinacea purpurea (Echi-p),* have been used to accelerate wound healing. However, the use of homeopathic preparations of these medicinal plants, in combination with PBM, is unknown. The objective of this study was to investigate the combined wound healing potential of these therapies *in vitro*. Various cell models were created *in vitro* in commercially available human skin fibroblasts (WS1). PBM was administered using a diode laser at a wavelength of 660 nm and an energy density of 5 J/cm^2. For homeopathic treatment, a 5% ethanolic complex containing *Calen*, *Hyper,* and *Echi-p* in 3cH potency was added to the culture medium. For combination therapy, cells were first treated with the homeopathic remedy and then irradiated. The controls included non-treated/irradiated cells and an alcohol control group. Cellular morphology was visualized 0, 24, and 48 h post-treatment. Post-treatment, cells were incubated for 48 h, and the levels of pro-inflammatory cytokines, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were determined by ELISA, cellular viability was determined by the Trypan Blue exclusion

assay and adenosine triphosphate (ATP) was determined by the luminescence assay, and cytotoxicity was determined by the lactate dehydrogenase (LDH) membrane integrity assay. Morphological changes showed that, individually, PBM and the homeopathic complex hastened cellular migration in diabetic wounded cells, and combining these therapies had an even greater effect. Cellular viability remained unchanged in both treatments, but the combination therapy increased ATP ($p = 0.05$). Cytotoxicity was decreased by the homeopathic complex ($p = 0.01$) and the combination therapy ($p = 0.004$). TNF-α levels remained unchanged with PBM and the homeopathic complex as independent treatment interventions but combining them increased the TNF-α levels in diabetic models ($p = 0.01$). PBM decreased IL-6 levels, indicating an anti-inflammatory effect ($p = 0.007$). These cellular changes indicated that combining PBM and a 3cH homeopathic solution of *Calen*, *Hyper,* and *Echi-p* is promising for treating diabetic foot ulcers.

Keywords
Photobiomodulation; wound healing; homeopathy; calendula; echinacea, hypericum; diabetic foot ulcers

1. Introduction

Wound healing is a well-regulated process that usually occurs promptly and follows a sequence of predictable events [1]. Various types of cells are recruited at the wound site by a succession of cytokine and signaling molecules [2]. Skin fibroblasts are varied cells that occur in the dermis [3] and play an important role in wound healing, including breaking down the fibrin clot and creating a new extracellular matrix (ECM) [4]. The wound healing process is disrupted in chronic wounds, which take more than three months to heal and display abnormal features, such as persistent inflammation [5]. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are pro-inflammatory cytokines produced by fibroblasts [6] and are produced at higher levels during the inflammatory phase of the wound healing process [2]. An example of a chronic wound is diabetic foot ulcer (DFU).

DFUs typically occur on the lower extremities of people suffering from diabetes mellitus (DM) [7]. They are highly prevalent, challenging to treat, and have a high recurrence rate [8]. DFUs are problematic as they increase the risk of lower-limb amputations and are a burden on health systems, of the patients and the caregivers [9]. The lifetime prevalence of DFUs is estimated to be as high as 25% among diabetics [10]. Various aetiological factors are implicated in DFUs, including peripheral neuropathy, increased vascular damage, and decreased repair, secondary to the metabolic abnormalities present in DM [11, 12]. Due to the difficulties in healing DFUs, novel treatment strategies are much needed, and photobiomodulation (PBM) is an attractive option.

PBM is an emerging therapy in the field of wound healing [13]. It refers to the use of low-powered light sources (typically in the visible red and near-infrared spectrum) to modify biological activity while avoiding tissue damage [14]. Its mechanism of action likely involves activation of the enzyme cytochrome c oxidase, thereby increasing ATP production and/or activation of light-sensitive ion channels [15]. PBM is a non-invasive and non-thermal therapeutic intervention that has been

proven to be effective for wound healing in both pre-clinical and clinical studies [16, 17]. Homeopathy is another potentially valuable treatment for DFUs.

Homeopathy is a complementary medical system founded in Germany in the late 18th century by Samuel Hahnemann and is based on the law of similars, i.e., on the principle that a substance, which produces certain symptoms in a healthy person, can cure those same symptoms in a diseased person [18, 19]. Plant extracts of *Calendula officinalis* L., *Hypericum perforatum* L., and *Echinacea purpurea* L. Moench have been shown to enhance wound healing capabilities [20-22]; however, research regarding homeopathic preparations of these remedies is limited [23-26]. Homeopathic medicines are different from herbal preparations in that they are used in different dilutions, known as potencies (see Figure 1). Dilutions are prepared from source materials, which are generally of plant, mineral, or animal origin [27]. Then, the material is processed by potentization, which involves trituration in lactose and/or serial dilution and succussion (vigorous shaking either by hand or mechanically) of the original substance [27]. For centesimal potencies, the substance is diluted in the solvent (usually a water/ethanol mixture) in a ratio of 1:100 and succussed to create the 1cH potency. The 2cH potency is created by similarly potentizing the solution with 1cH potency. This process is repeated until the required potency is achieved. Centesimal potencies are denoted with the letter 'c', while the 'H' indicates that the Hahnemannian method was used [18].

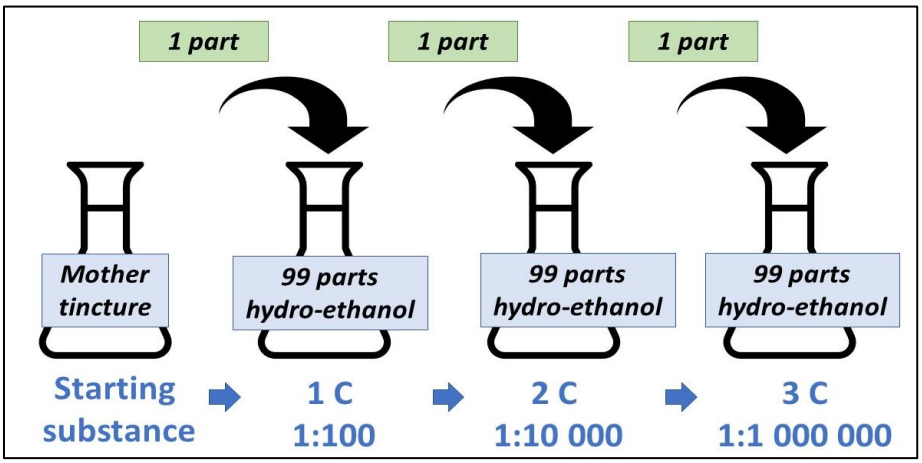

Figure 1 Shown is the schematic representation of the potentization process of homeopathic remedies using the centesimal (C) scale for illustration.

The 3cH potency was chosen for this study as it is considered a 'low' potency, meaning that it contains molecules of raw plant matter, unlike potencies above 12cH. Furthermore, low potencies are typically used in 'clinical homeopathy', which is a homeopathic treatment approach where remedies are prescribed according to the presenting clinical condition, rather than being individualized to the patients' mental, emotional, and physical symptoms, thus making it ideal for targeted healing of DFUs.

The objective of this study was to assess the effects of PBM combined with an ethanolic homeopathic complex containing *Calen, Hyper,* and *Echi-p* in a 3cH potency, in an *in vitro* diabetic wounded cell model.

2. Materials and Methods

2.1 Cell Culture and Models

Materials were supplied by Sigma-Aldrich (Gauteng, South Africa) unless stated otherwise. Commercially available human skin fibroblasts (WS1, ATCC® CRL-1502™) were cultured in 75 or 175 cm^2 culture flasks (431464U and 431466, Corning®, The Scientific Group, Gauteng, South Africa) in Minimum Essential Medium (MEM, M2279), supplemented with 10% fetal bovine serum (FBS, 10499–044, Gibco®, ThermoFisher Scientific, Gauteng, South Africa), 1 mM sodium pyruvate (S8636), 2 mM L-glutamine (G7513), 0.1 mM non-essential amino acids (NEAA, M7145), 1% Penicillin-Streptomycin (100 units Penicillin and 0.1 mg/mL Streptomycin, P4333), and 1% Amphotericin-B (2.5 µg/mL, A2942). Cells with passage numbers between 11 and 16 were used in the experiments. For the experiments, cells were seeded at a concentration of 6 X 10^5 into 3.4 cm diameter tissue culture plates and allowed to attach overnight.

The models used were normal (N), normal wounded (NW), and diabetic wounded (DW). Wounded models were created by performing the scratch assay, which involves creating a central cell-free area by scraping with a 1 ml sterile pipette [28]. This *in vitro* method for wound healing is well-established in the field as a method to study cell migration and has several advantages, such as being easy and relatively cost-effective to perform [29]. Cells were allowed to acclimatize for 30 min before any further treatment protocols were performed. Non-wounded (i.e., normal) cells were used as controls. A diabetic cell model was created by continually growing cells in a complete medium supplemented with 17 mM/L D-glucose [30]. These models have been extensively used and are well-established at our research centre center [31-33].

2.2 Treatment Protocols

Treatment consisted of either PBM, homeopathic treatment, or both. PBM groups were irradiated in the dark from above with visible red light at a wavelength of 660 nm with an energy density of 5 J/cm^2 (11 mW/cm^2, 9.1 cm^2 spot size, 454 sec) as previously described [33], but with 2 ml culture medium added to the plates. Energy densities of 3–5 J/cm^2 were reported in a review to yield optimal results for healing diabetic wounds [34]. Additionally, our research group had optimized a protocol, where 660 nm wavelength of light was used and showed favorable outcomes [33]. The homeopathic treatment involved adding the homeopathic solution (30 µL) to plates containing 2 ml complete medium.

A reputable and registered homeopathic manufacturing company (Fusion Homeopathics CC, Randburg, South Africa) obtained the mother tinctures of the three plants from Gehrlicher Pharmaceutical Extracts GmbH (certificates of the analysis are available on request). Fusion Homeopathics potentized the tinctures separately to a 3cH homeopathic potency according to standardized homeopathic manufacturing procedures and then combined them into a complex. An alcohol control group was included, where 5% ethanol (EtOH), prepared with distilled water, was added to the culture medium in normal wounded cells (final concentration of 0.074% EtOH). This group was included to ensure that the vehicle of the complex did not cause undue cellular stress. In models receiving both homeopathic and laser (PBM) treatment, the homeopathic treatment was added first and then irradiated as described above.

A preliminary experiment was performed to determine which solvent to use for a homeopathic complex. A 3cH homeopathic complex containing equal parts of *Calen*, *Hyper,* and *Echi-p* was prepared in distilled water (dH2O) and in different concentrations of ethanol (2.5%, 5%, and 10% EtOH) according to the German Homoeopathic Pharmacopoeia (GHP) [35] following the method GHP 3A for all three remedies. All three components were prepared separately up to the 2 cH level by a homeopathic manufacturer (Fusion Homeopathics CC, Randburg, South Africa), and the last potentization step was performed with the three components combined at equal quantities. These solutions were tested on normal wounded cells. Determination of the cellular morphology, viability (Trypan Blue exclusion assay and ATP experiments), and cytotoxicity (LDH) were performed as described below. Based on the results, 5% ethanol was as the solvent to be used in the homeopathic treatment protocols.

2.3 Cellular Morphology

An inverted light microscope (CKX41, Olympus, Wirsam Scientific, Gauteng, South Africa) connected to a digital camera (SC30, Olympus) was used to capture images of the cell monolayer at 0 h (directly after treatment, if any), 24 h, and 48 h after treatment to observe morphological changes. The AnalySIS getIT software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) was used, and the images were captured using the 4X objective.

2.4 Trypan Blue Exclusion Assay

In this assay, non-viable cells stain blue as their damaged membranes cannot prevent the blue dye from freely entering the cell; viable cells remain colorless. Cells were detached by adding 500 µl of pre-warmed TrypLE™ Select (12563–029, Gibco®, ThermoFisher Scientific, Gauteng, South Africa) to the culture plates and incubated for 5 min. Cells were resuspended in 1 ml of serum-free medium, and 10 µl of 0.4% Trypan blue (T6146) was added to 10 µl of the cells. The mixture was gently mixed by pipetting, and then, 10 µl of cells were added to each chamber of a re-usable cell counter slide. The Invitrogen Countess® II FL (ThermoFisher Scientific, Gauteng, South Africa) was used to count cells and determine the percentage of live cells; an average of two readings was used for the analysis.

2.5 Lactate Dehydrogenase (LDH) Membrane Integrity Assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1782, Promega, Anatech, Gauteng, South Africa) was used to quantify LDH. The amount of LDH released reflects the degree of membrane damage, which indicates the level of cellular toxicity. The reconstituted substrate mix (50 µl) was added to each well of a clear 96-well plate. Complete cell culture medium (50 µl), which had not been used to culture cells, was added to two wells with the substrate mix to serve as a background control. The culture medium (50 µl) from the experimental plates was added to the rest of the wells containing the substrate mix and gently mixed. The plate was covered with a foil and incubated in the dark at room temperature for 30 min. Stop Solution (50 µl) was added to each well, and absorbance was detected by using a multiplate reader (Victor^3, 1420 Multilabel counter, Perkin-Elmer, Gauteng, South Africa) at 490 nm. The average background reading of the control was subtracted from all the values recorded in the treatment groups.

2.6 Adenosine Triphosphate (ATP) Luminescence Assay

The CellTiter-Glo® 3D Cell Viability Assay (G9682, Promega, Anatech, Gauteng, South Africa) was used to determine metabolically active or viable cells. The intensity of the luminescent signal produced is proportional to the amount of ATP present, which in turn is directly proportional to the number of viable cells present in the culture. Cells were detached as described above. Complete culture medium (50 µl), which had not been used to culture cells in, was added to two wells of an opaque-walled 96-well plate to serve as a background control. Then 50 µl of the cell suspension was added to the designated wells, and 50 µl of the reagent was added to all the wells. The plate was covered with foil and placed on an orbital shaker (Polymax 1040, Heidolph Instruments, Schwabach, Germany) for 10 min. Luminescence (in Relative Light Units or RLU) was measured using a multiplate reader (Victor^3, 1420 Multilabel counter, Perkin-Elmer, Gauteng, South Africa). The average background reading of the control was subtracted from all the values recorded in the treatment groups.

2.7 Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α) ELISA

The enzyme-linked immunosorbent assay (ELISA) is a plate-based assay, which can quantify levels of target molecules such as proteins in samples by binding to specific antibodies. Commercially available pre-coated kits were used to quantify pro-inflammatory cytokines IL-6 (E-EL-H0102, Elabscience®, Biocom Africa, Gauteng, South Africa) and TNF-α (E-EL-H0109, Elabscience®, Biocom Africa, Gauteng, South Africa) released from the cells in culture media. The methodology followed for both the assays was identical and was as per the manufacturer's instructions. Culture media samples obtained previously were prepared by thawing and centrifuging for 20 min at 1,000 g, and the supernatant was decanted and used for the ELISA. The manufacturer's instructions were used to determine the protein concentration by comparing optical density values to a known protein concentration obtained from the eight-point serial standard curves.

2.8 Statistical Analysis

Data points are represented as means ±standard deviations. Experiments were repeated three times (n = 3). The student's t-test was used to determine differences within groups, and one-way ANOVA with the Tukey post hoc test was used to detect differences between group means using SPSS 25.0 and Microsoft Excel. The differences between or among groups were considered to be statistically significant for p-values < 0.05.

This was an *in vitro* study conducted on a commercially available cell line (ATCC). This research project was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Johannesburg (Ethics number REC-01–19–2019).

3. Results

3.1 Preliminary Experiment

In the preliminary experiment, cellular responses (morphology, cellular viability, and cytotoxicity) were determined for the 3cH homeopathic complex prepared in three different ethanol

concentrations (2.5%, 5%, and 10%) and compared to a distilled water control (dH2O) in a normal wounded cell model.

Morphological changes were examined at 0, 24, and 48 h. The central scratch, or 'wound', was visible as a cell-free area in the center of the tissue culture dish with cells on either side. Cells could be seen migrating and moving toward the central scratch over the 48 h incubation period (Figure 2). Cells treated with 5% EtOH solution had more cells present in the central scratch compared to those treated with 2.5% and 10% EtOH and the dH2O control, indicating increased migration. Cellular viability was determined by Trypan blue staining and ATP luminescence 48 h after adding the homeopathic complex. No statistically significant differences were detected in percentage cell viability and ATP luminescence between the controls (dH2O) and the 2.5%, 5%, and 10% EtOH solutions (Table 1). Cytotoxicity was determined by measuring the LDH levels in culture media, which indicated damage to the cell membrane. There was a significant increase in the LDH released in all three EtOH groups (2.5%, 5%, and 10%, respectively) compared to that released in the control (dH2O) group (Table 1).

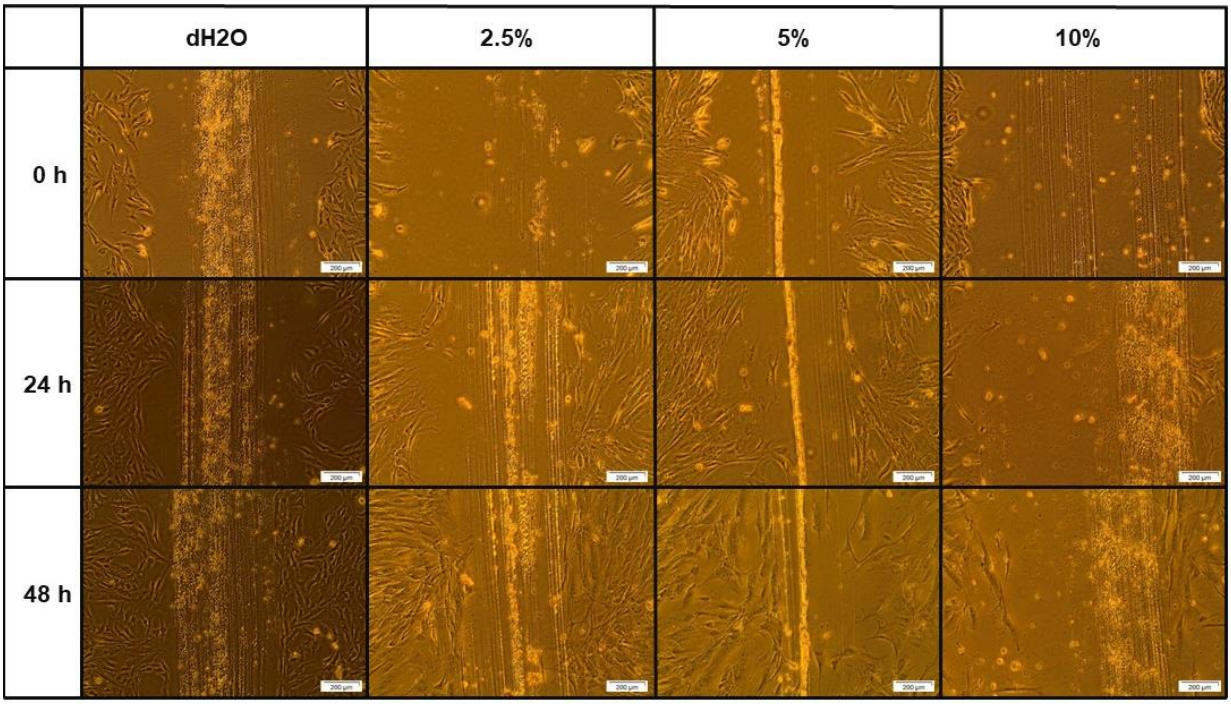

Figure 2 Shown is the morphology of normal wounded cells treated with the homeopathic complex prepared in different ethanol (EtOH) concentrations. Cells can be seen migrating towards the central scratch (wound), with faster migration for treatment with the homeopathic complex in 5% EtOH. Scale bars = 200 µm.

Table 1 Cell viability (percentage viability and ATP luminescence) and cytotoxicity (LDH membrane integrity) of the cells post-treatment at 48 h. The models assessed were for normal wounded cells to which the homeopathic complex, prepared in distilled water (dH2O), 2.5% ethanol (2.5% EtOH), 5% ethanol (5% EtOH), and 10% ethanol (10% EtOH), was added. Results are shown as the mean ±SD, and the statistical significance is for the comparisons between the EtOH treatments and distilled water; *$p < 0.05$ and **$p < 0.01$; RLU = Relative light units.

Solvent	Trypan blue (% viability)	ATP luminescence (RLU)	LDH membrane integrity ($A_{490\ nm}$)
dH2O	77 ±7	1404130 ±59958	0.195 ±0.025
2.5% EtOH	77 ±4	1376846 ±176158	0.510 ±0.073*
5% EtOH	74 ±5	1245430 ±88949	1.141 ±0.151**
10% EtOH	77 ±7	1214010 ±95667	0.573 ±0.043**

Based on the above results and the observed increase in cellular migration, EtOH concentration of 5% was selected as the solvent for the homeopathic complex for further studies, despite increased levels of cytotoxicity (which was observed at all three concentrations). This was similar to the results of the study by Bresler et al. [26], who had found that an alcohol concentration of 5% was preferred as it produced the greatest degree of cell migration with minimal cell death at 48 h.

3.2 Cellular Morphology

Cellular responses to the various treatment conditions were determined qualitatively by comparing morphology at 0, 24, and 48 h. In diabetic and normal models, cells maintained their characteristic spindle shape, with minimal detachment from the culture dish or rounding of cells that indicates cellular stress or death. Normal cells, which were not wounded (Figure 3), did not show any morphological differences following laser irradiation, homeopathic treatment, or combination treatment, and the monolayer remained intact.

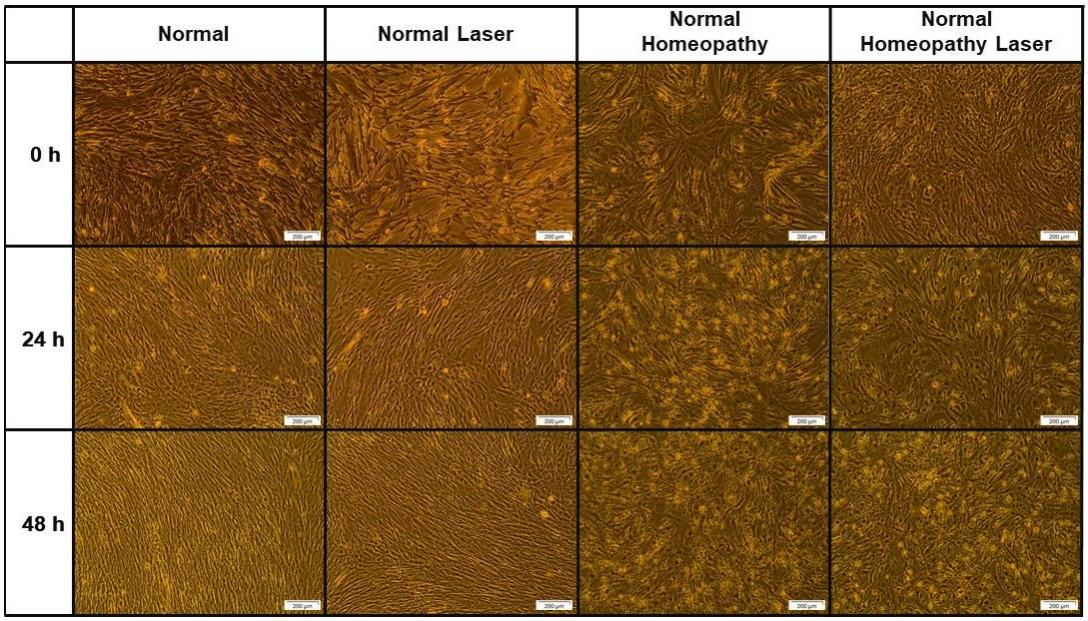

Figure 3 Shown is the morphology of normal cell models. Treatment with laser irradiation and/or homeopathic complex did not change the morphology of WS1 human skin fibroblast cells. Cultures remained confluent over the 48 h period. Scale bars = 200 µm.

In the normal wounded models (Figure 4), laser irradiation-induced a dramatic increase in cellular migration compared to the migration in the controls. Some degree of cellular debris was visible in all models; however, the monolayer was still intact, and the cultures were confluent. Homeopathic treatment also increased wound closure, but to a lesser degree than laser irradiation. Combining laser irradiation and homeopathic treatment improved migration to a greater extent than migration in either condition in isolation. In the diabetic wounded models (Figure 5), there was an overall reduction in the migration of cells compared to the migration of cells in the normal wounded models. As in the normal wounded models, laser irradiation and homeopathic treatment improved cellular migration in the models compared to cellular migration in control; moreover, there was a mild synergistic effect in combination therapy.

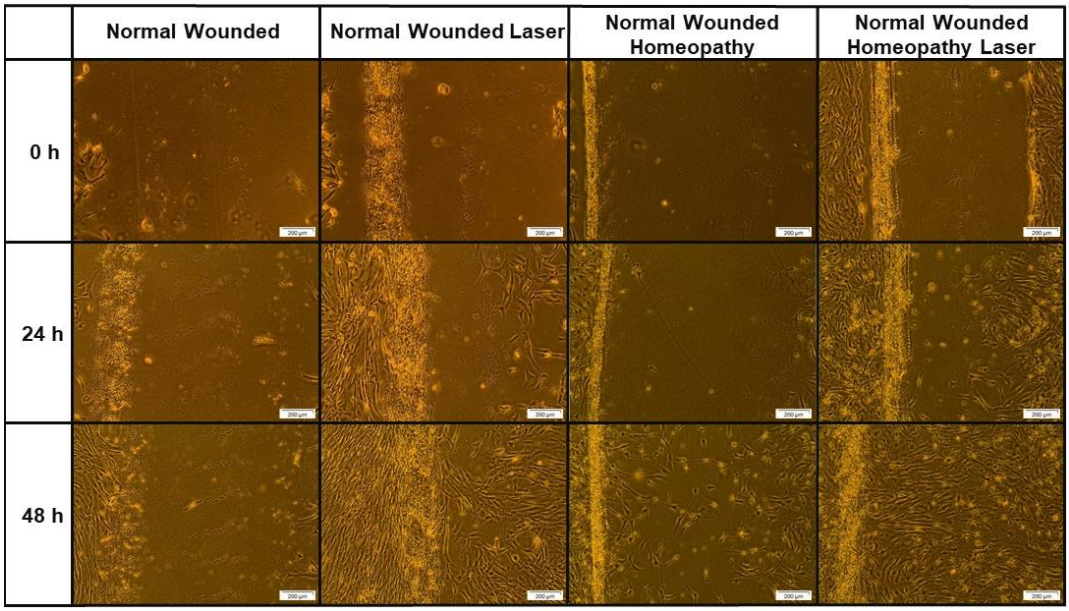

Figure 4 Shown is the morphology of normal wounded cell models. Laser irradiation, homeopathic treatment, and a combination of the two accelerated cellular migration into the central scratch, with almost complete closure at 48 h in cells treated with both laser irradiation and homeopathic complex. Scale bars = 200 µm.

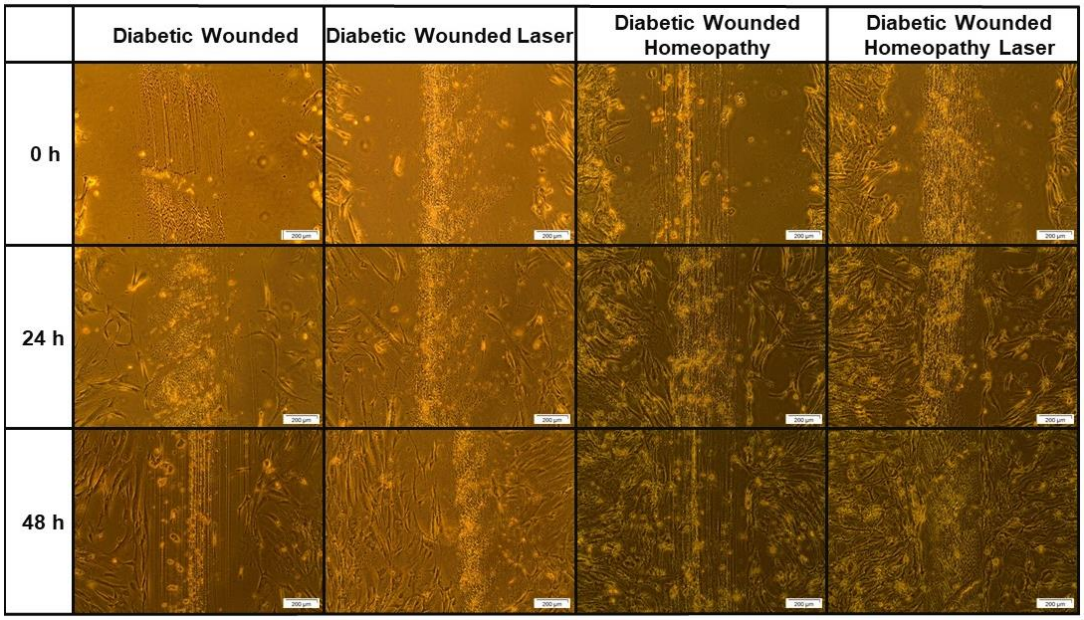

Figure 5 Shown is the morphology of diabetic wounded cell models. Laser irradiation, homeopathic treatment, and a combination of the two increased cellular migration into the central scratch over the 48 h period. Scale bars = 200 µm.

3.3 Cellular Viability

Cellular viability was assessed by the Trypan blue exclusion assay and the ATP luminescence assay 48 h post-treatment (Table 2). Results for the Trypan blue exclusion assay revealed lower viability in laser irradiated normal cells compared to non-irradiated normal controls. Combining laser

irradiation and homeopathic complex decreased viability of the normal wounded and diabetic wounded cells.

Table 2 Cell viability (percentage viability and ATP luminescence) and cytotoxicity (LDH membrane integrity) of cells post-treatment (homeopathic treatment and/or laser irradiation at 660 nm with 5 J/cm^2) after 48 h. The models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic complex (NH), normal cells treated with homeopathic complex and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic complex (NWH), normal wounded cells treated with homeopathic complex and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic complex (DWH), and diabetic wounded cells treated with homeopathic complex and irradiated (DWHL). An alcohol control model was included, which consisted of normal wounded cells to which 5% EtOH was added (NW 5% EtOH). Results are shown as mean ±SD, and the statistical significance, compared to untreated control cells (non-irradiated and no homeopathic complex; N, NW, and DW, respectively) within each model, is denoted by *p < 0.05, **p < 0.01, and ***p < 0.001; RLU = relative light units.

Model	Trypan blue (% viability)	ATP luminescence	LDH membrane integrity ($A_{490\ nm}$)
NW 5% EtOH	76 ±5	247123 ±34160**	1.209 ±0.027***
N	78 ±4	383670 ±25847	0.420 ±0.011
NL	72 ±4*	1328944 ±183651*	0.385 ±0,026
NH	78 ±1	498918 ±40464*	0.597 ±0.061*
NHL	81 ±3	212911 ±29262*	0.231 ±0.015**
NW	79 ±2	402066 ±61983	0.542 ±0.026
NWL	76 ±3	345657 ±97757	0.464 ±0.054
NWH	74 ±5	1245430 ±88949***	1.141 ±0.151**
NWHL	73 ±3**	292068 ±25556*	0.211 ±0.063**
DW	75 ±4	429298 ±31315	0.561 ±0.006
DWL	80 ±4	308337 ±136652	0.809 ±0.060*
DWH	75 ±6	441837 ±64395	0.227 ±0.051**
DWHL	66 ±4*	515377 ±40414*	0.300 ±0.039**

ATP levels were lower in normal wounded cells treated with 5% EtOH compared to the levels in the control cells, suggesting that the solvent of the complex caused some degree of toxicity and negatively affected ATP production. In normal models, laser irradiation and homeopathic treatment independently increased ATP levels compared to the ATP levels in controls, but the combination treatment decreased ATP levels. In normal wounded models, homeopathic treatment increased ATP levels, and the combination treatment decreased ATP levels. In the diabetic wounded models, the combination treatment caused ATP levels to increase – the opposite of the effect observed in normal wounded models. No differences were detected in cellular viability between the normal wounded and diabetic wounded cells, based on ANOVA.

3.4 Cytotoxicity

Homeopathic treatment increased cytotoxicity in the normal models (Table 2), while a combination of homeopathic treatment and laser irradiation decreased cytotoxicity, and laser irradiation did not produce any significant changes. In the normal wounded models, there was a similar effect, where the homeopathic treatment increased cytotoxicity and a combination of irradiation and homeopathy decreased it; no significant effect was found with laser irradiation alone. In the diabetic wounded models, the opposite pattern was observed, where laser irradiation increased cytotoxicity, the homeopathic treatment decreased it, and a combination treatment decreased cytotoxicity. No differences were detected in cytotoxicity between the normal wounded and diabetic wounded cells, based on ANOVA.

3.5 Interleukin-6 and Tumor Necrosis Factor-Alpha

The results of the ELISA for IL-6 are shown in Figure 6. In normal models, homeopathic treatment and homeopathic treatment combined with laser irradiation increased IL-6 levels. In normal wounded models, laser irradiation significantly decreased IL-6 levels, while the homeopathic treatment alone increased IL-6. The combination treatment of normal wounded cells decreased IL-6 levels. Laser irradiation of diabetic wounded models significantly decreased IL-6 levels; however, homeopathy combined with laser irradiation increased IL-6 levels, the opposite of the effect seen under these treatment conditions in the normal and normal wounded cells.

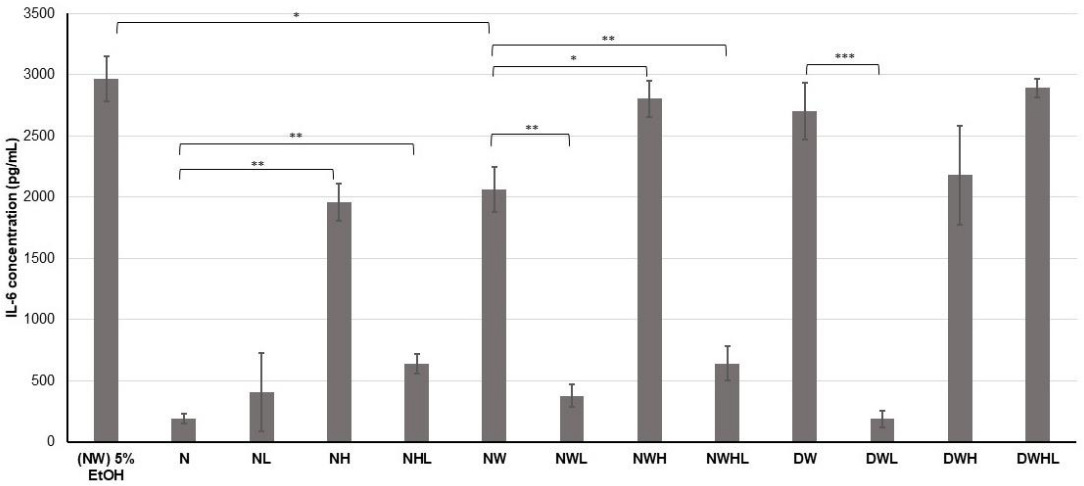

Figure 6 Shown are the IL-6 cytokine levels determined from the culture medium. Statistically significant differences are denoted by *p < 0.05, **p < 0.01, and ***p < 0.001, compared to non-irradiated control cells. Bar plots show mean ±SD

Homeopathic treatment increased inflammation, as determined by IL-6 levels, except in the diabetic wounded cells. No differences were detected in IL-6 levels between normal wounded and diabetic wounded cells, based on ANOVA.

TNF-α levels remained unchanged for many of the treatment conditions, compared to the levels in the control cells (Figure 7). Homeopathic treatment of normal cells in isolation and combined with laser irradiation decreased TNF-α levels. In the normal wounded models, the homeopathic

treatment decreased TNF-α levels as well. In the diabetic wounded models, the homeopathic treatment, combined with laser irradiation, significantly increased TNF-α levels. The levels were decreased by the application of 5% ethanol, the opposite of the effect seen in IL-6. No differences were detected in TNF-α levels between normal wounded and diabetic wounded cells, based on ANOVA.

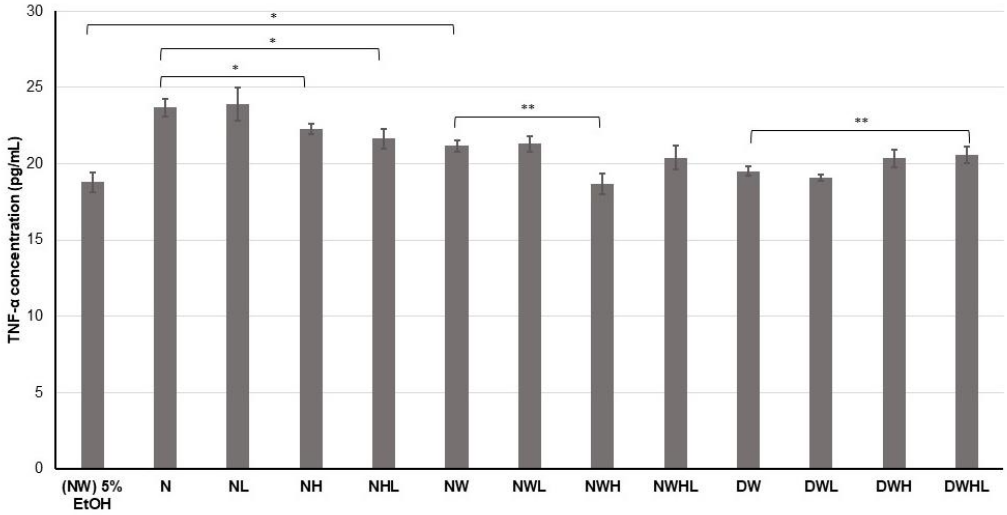

Figure 7 Shown are the TNF-α cytokine levels determined from the culture medium. Statistically significant differences are denoted by *p < 0.05 and **p < 0.01, compared to the TNF-α levels in non-irradiated control cells. Bar plots show mean ±SD.

4. Discussion

The decreased migration of diabetic cells compared to the migration of the normal wounded models was expected since diabetic cells showed decreased cellular migration, which contributed to lesser healing of chronic wounds [5]. Other studies had also found an increase in cellular migration following irradiation [26, 33, 36]. It was shown that laser irradiation at 660 nm and 5 J/cm^2 activated the epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) loop leading to the activation of the JAK/STAT signaling pathway, which in turn promoted cellular migration and proliferation [33]. To our knowledge, the specific combination of homeopathic remedies had not been tested in the context of wound healing; however, studies on herbal extracts of *Calen* [37], or those that used a homeopathic complex containing *Calen* and *Hyper* [25], also showed increased cellular migration.

Other studies had also found that PBM could increase ATP levels in fibroblasts when irradiated with 660 nm, as was found in this study [33, 38]; this effect was also found for lymphocytes irradiated with lasers between 632.8 and 830 nm [39]. We found different responses of ATP levels to irradiation in different models; however, the effect seen in the diabetic wounded model with combination treatment was encouraging. Homeopathic treatment increased cellular viability in normal and normal wounded models; it was also found by Bresler et al. [26] using *Calen* 3cH. The Trypan blue exclusion assay and ATP luminescence assay both measure cellular viability, but the principle of the two assays is different. The Trypan blue exclusion assay relies on the integrity of the

cellular membrane, while the ATP luminescence assay measures ATP based on mitochondrial activity.

Bresler et al. [26] found decreased cytotoxicity with 3cH *Calen,* also made in 5% EtOH, but with a lesser amount of the complex added to the plates, which could explain the differences in the results. Other studies found that irradiation at 5 J/cm^2 caused no cytotoxicity in WS1 cells (also used in this study), which supported our findings in the normal and normal wounded cells [40]. Ethanol control experiments revealed significantly increased LDH levels compared to the levels in the control normal wounded cells, thus indicating cytotoxicity in the ethanol-treated normal wounded cells. Alcohol concentrations, as low as 1.5%, were found to be cytotoxic to human hepatoma cells [41]. However, other studies had found signs of damage only at 8% ethanol after 3 h of exposure in an experiment using 4–12% ethanol in the gastric epithelial cells of rats. The degree of damage was dependent on the concentration and duration of exposure to ethanol [42]. This indicates that the cell line is probably an important factor in cellular cytotoxicity studies involving ethanol.

Other studies had found that extracts of *Calen* [43], *Hyper* [44], and *Echi-p* [45] inhibited IL-6 release. These results indicated that PBM decreased inflammation after 48 h, thus demonstrating its well-known anti-inflammatory effect [46, 47]. Hyperglycemic obese mice had smaller areas of inflammatory infiltrates after PBM treatment [47]. A review by Hamblin [46] summarized how IL-6 and other pro-inflammatory markers are reduced in various disease states. Direct comparison is difficult due to the specific combination of these three substances used in this study, and the fact that it was prepared as a 3cH homeopathic solution, and thus, was more diluted than herbal extracts. Additionally, the increase in IL-6 could be ascribed to effects of the 5% ethanol solvent, as the 5% alcohol control had significantly higher IL-6 levels compared to the levels in the control normal wounded cells. The paradoxical effect of the combination treatment in diabetic wounded cells compared to the treatment in the normal and normal wounded combination group was interesting and could be ascribed to the diabetic cells being grown under hyperglycaemic conditions. When diabetic wounded control cells were compared to normal wounded control cells, the level of IL-6 was found to be higher, and this effect was ameliorated with laser irradiation but not with homeopathic intervention alone.

Other studies had found that the extracts of *Calen* and *Hyper* inhibited the TNF-α response [43, 48], while *Echi-p* effectively inhibited TNF-α in the early phase but prolonged it in the later phases of inflammation [49]. The time-dependent nature of cytokine responses should be considered while interpreting the results. The 48 h time point used in this study might have been too late to detect meaningful TNF-α changes as they are typically affected much earlier than IL-6 levels, and early changes might have been missed. IL-6 and TNF-α levels are usually detectable immediately after injury, with IL-6 levels peaking within 30 h [50, 51], and TNF-α levels reaching their maximum within 4 h after injury [52, 53]. Some studies [54] have found that laser irradiation in the visible red range (at 636 nm, with 5 J/cm^2) in similar fibroblast cell models (normal wounded and diabetic wounded) does not show any significant changes in TNF-α 24 h post-irradiation; however, they did find a decrease in normal wounded cells 1 h post-irradiation.

The positive effect on cellular migration and ATP levels observed in the combination therapy group might be explained by various cellular mechanisms, although it is not yet clearly determined. Light absorption by chromophores in response to PBM triggers various responses, including activation of calcium-gated ion channels and downstream effects such as protein transcription [46]. The mechanism of action of homeopathic treatment is unclear, but studies using electron

microscopes, electron diffraction, and DNA microarray investigations have confirmed the presence of nanoparticles in homeopathic medicines, which might act in unique ways that are not yet understood [55].

Future studies on the combination of PBM and homeopathic remedies should be performed for a longer duration, with effects recorded at various time intervals, to determine if this combination therapy affects pro-inflammatory cytokines IL-6 and TNF-α. Care should be taken in future studies that the control solvent is succussed or vigorously shaken to the same extent as the remedies. This is because the physical composition of liquids changes by succussion due to the formation of nanobubbles and changes to the air-liquid interface [56], as well as the presence of silica and other nanoparticles from glassware [57]. Succussion alone can change the expression of some inflammatory parameters [58], which strongly suggests the inclusion of a succussed control. A limitation of this study was using ethanol as a solvent, as it independently affected cells. To address this issue, sterile water could be used as a solvent in the final dilution step of manufacturing homeopathic remedies to provide clearer results in future experiments. Additionally, an alcohol control consisting of succussed hydro-ethanol of the same concentration as the vehicle could be included as a control since it acts as a remedy and needs to be included to ascertain the actual effects of the homeopathic preparation *in vitro.*

5. Conclusions

In this study, the wound healing potential of PBM at 660 nm with a fluence of 5 J/cm^2 and a homeopathic preparation containing 3cH *Calen, Hyper,* and *Echi-p* was evaluated as independent therapies as well as a novel combination treatment protocol for DFU. PBM increased cellular migration to the wounded area and decreased cytotoxicity and the levels of the pro-inflammatory cytokine IL-6 in diabetic wounded cells. A homeopathic preparation containing 3cH *Calen, Hyper,* and *Echi-p* also improved cellular migration and decreased cytotoxicity in diabetic wounded cells. A combination therapy involving homeopathic treatment, followed by laser irradiation at 660 nm, increased cellular migration. Additionally, a synergistic effect was found in the combination therapy compared to the effects of either therapy in isolation. Furthermore, the combination therapy decreased cytotoxicity and increased cellular viability.

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Author Contributions

NH and JP are responsible for the study design. JW performed the assays and molecular analyses and statistical evaluations with the help of a statistician. NH, JP and JW participated in data analysis and interpretation. All authors wrote and approved the final manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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