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Research Article
Healing Effect of *Plantago major* and Photodynamic Therapy Combination on Skin Wounds

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Abstract

Background and objective: Current strategies to address wound care include both traditional and modern options. Medicinal plants, as a part of traditional medicine and photodynamic therapy, as a modern option, have shown promising effects in wound care and repair. In this study, a combination of *Plantago major* and laser therapy might have more effective wound healing effects was hypothesized. **Materials and Methods:** To examine this, *P. major* extract was prepared through the maceration method. Human fibroblasts were harvested in FBS and antibiotic-contained DMEM. Upon reaching 70-80% confluency, cells were seeded in a 96-well plate and then treated with *P. major* (different concentrations), laser (980 nm) and *P. major*-laser combination. To determine the cell viability percentage, an MTT assay was performed. A scratch assay to investigate HFFF2 migration after treatment with *P. major*, laser and their combination was also carried out. The expression of IL-6 and TNF-α as pro-inflammatory cytokines and VEGF as a growth factor were analyzed using a real-time PCR technique. **Results:** Current findings showed that 160 μg mL^{-1} *P. major* as well as the combination of 160 μg mL^{-1} *P. major* and laser posed no cytotoxicity to HFFF2 cells. Scratch assay results indicated that the *P. major* and laser combination could effectively increase HFFF2 migration ability as compared to control cells. An upregulated VEGF, IL-6 and TNF-α gene expression in cells treated with the combined *P. major* and laser was also found. **Conclusion:** The evidence from this study suggested that the combination of traditional and modern wound repair approaches could result in more favorable outcomes.

Key words: Wound healing, *Plantago major*, photodynamic therapy (PDT), Human Fibroblast (HFFF2), migration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Wounds are hallmarks of disrupted cellular, anatomical and functional integrity of living cells and tissues, which commonly arose from electrical, physical, chemical or microbial threats[1]. Wound healing is defined as a highly sophisticated process of regeneration and reconstruction of damaged cells and tissues[1]. The process of wound healing proceeds through four distinct and overlapping phases including hemostasis, inflammation, cell proliferation and migration and tissue remodeling[2,3]. To put it more sharply, a normal response to wound healing is a sequence of integrated cellular and biochemical events that ideally result in scar formation in a short time[4,5]. Interestingly, wound splinting process can be facilitated by medicinal herbs that are an indispensable part of traditional medicine.

Plantago major is a perennial plant, which belongs to the Plantaginaceae family and is native to most of Europe and Asia as well as North Africa and North America[6]. The medicinal properties of *P. major* are attributed to its various bioactive compounds such as flavonoids, alkaloids, iridoids, phenolic acids, terpenoids, polysaccharides, fatty acids and vitamins[7,8]. In traditional medicine, this plant has been extensively administered in a number of diseases related to skin, circulation, reproduction, respiratory organs, digestive organs, infections and tumors[7-9]. Interestingly enough, it has been suggested that *P. major* exerts its wound healing effect through different mechanisms including antioxidant, anti-inflammatory and antimicrobial functions and stimulation of fibroblast proliferation[10-12].

Photodynamic therapy (PDT) is a modern non-invasive technique, which plays a major role in the treatment of cancers, some infections and other diseases[13]. Several studies have demonstrated that PDT can enhance wound healing through bacterial inactivation in the wound site[14-16]. It is also important to note that PDT increases or decreases inflammation (depending on the administered dose), increases elastin and collagen production, induces fibroblast proliferation and upregulates TGF-β and metalloproteinase gene expression[17]. To the best of our knowledge, hitherto, there is no available data on the effect of *P. major* and PDT combination on the wound healing process. Hence, this study investigated the role of *P. major* and PDT combination to improve wound healing procedures.

MATERIALS AND METHODS

Study area: Hamadan University of Medical Sciences, University of Sulaimani and Bakrajo Technical Institute Sulaimani Polytechnic University.

Plant materials and extraction: The aerial parts *P. major* were collected from the Kurdistan and Herbarium number 32 was awarded to the Herbarium of Hamadan University of Medical Sciences. Extraction of the plant was performed through the maceration method (soaking in the solvent at room temperature). For this purpose, 300 g of the plant were weighed and then extracted in hydro-alcoholic solvents (70% ethanol and 30% water). Extraction was done for 3 days with a solvent to tissue ratio of 4:1. Next, the filtration method was used to separate the obtained *P. major* extract. The prepared extract was re-filtered through a high-purity filter paper and then dried using a rotary evaporator under a vacuum at 45°C. The obtained extract was stored at -40°C for further experiments[11].

Cell culture: Human Caucasian Fetal Foreskin Fibroblast (HFFF2) cells were obtained from Pasteur Institute (Tehran, Iran). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM/LG, GIBCO) enriched with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humid atmosphere containing 5% CO_2. Cultured cells were monitored every 48 hrs until reaching 70-80% confluent.

Laser treatment: The PDT system used in this treatment group was a 980 nm diode laser light (0.5 W, 5 J/cm^2, Pioon, China). At first, HFFF2 cells were seeded in a 96-well plate. Then cells were exposed to a 980 nm diode laser (0.5 W, 5 J/cm^2, Pioon, China) in a dark place for 10 sec.

***Plantago major* extract treatment:** The HFFF2 cells were seeded in a 96-well plate and incubated overnight at 37 After incubation, cells were treated with a concentration range of 0, 1, 10, 20, 40, 80, 100, 160, 320, 640 and 1000 μg mL^{-1} *P. major* for 24 and 48 hrs.

Combined laser and *P. major* extract treatment: First, cells were seeded in a 96-well plate and incubated at 37 for 24 hrs. Cultured cells were treated with 160 μg mL^{-1} *P. major* and then exposed to 980 nm diode laser for 10 sec. Next, the plate was placed in an incubator at 37°C for 24 and 48 hrs.

MTT assay: After each treatment time slot, cells in all three groups were treated with 10 μL tetrazolium salt (0.05 mg mL^{-1}) and incubated at 37°C for 4 hrs. After incubation, the MTT-contained culture medium was discarded and 100 μL Dimethyl sulfoxide (DMSO) was added, followed by 10 min of incubation in a dark place. Then needle-shaped formazan crystal formation was quantified by measuring absorbance at 570 nm using a microplate reader ELISA (company, country).

Wound healing scratch assay: To perform the scratch assay, the fibroblasts were seeded into a 12-well plate at a density of 1×10^5 cells/well and incubated overnight at 37. After incubation, a scratch was made in a straight line with the aid of a 200 µL pipette tip. Cells were washed with phosphate-buffered saline (PBS) and then were treated with different concentrations of *P. major* (0, 1, 10, 20, 40, 80, 100, 160, 320, 640 and 1000 µg mL^{-1}) for 24 and 48 hrs. The second group of cells was subjected to 980 nm laser for 10 sec and the third group was treated with the combination of 160 µg mL^{-1} *P. major* and laser. The group of cells that underwent no treatment was regarded as control group. Changes in the scratch area were monitored using an inverse light microscope (ECLIPS 80i with WG filter, Nikon, Tokyo, Japan) equipped with a digital camera. Images from the scratch area were taken during 6, 12, 18, 24 and 30 hrs[18]. The wound healing percentage was measured according to the below mentioned equation:

$$\text{Wound healing (\%)} = \frac{\text{Scratch area at time 0-scratch area at specific time}}{\text{Scratch area at time 0}}\times100$$

RNA extraction, cDNA synthesis and real-time PCR: Total RNA was extracted from HFFF2 cells according to the instruction of the Trizol one-step method regarding mRNA and their purity and concentration were measured. The cDNA synthesis was followed by the instruction of the TaKaRa Reverse Transcription System. Real-time PCR was conducted using SYBR green master mix high ROX™ (Ampliqon, Denmark) with an initial denaturation at 94°C for 30 sec and the subsequent cycles of 30 sec at 60°C and 30 sec at 37°C. The primer sequences for studied genes were as follows: GAPDH forward VEGF (Vascular Endothelial Growth Factor) forward TCACCATGCAGATTATGCGGA and reverse CACGCTCCAGGACTTATACCG, TNF-α forward GGGCCTGTAC CTCATCTA and reverse AGACCCCTCCCAGATAGATG, IL-6 forward TTCGGTCCAGTTGCCTTCTC and reverse TGTTTTCTG CCAGT GCCTCT. The calculation was performed using REST analysis software (QIAGEN, Germany). The GAPDH gene was used as the internal reference. The expression of VEGF, TNF-α and IL-6 genes was analyzed relative to the GAPDH expression as internal reference gene. Real-time PCR reactions were performed in triplicate, while running all samples in duplicates.

Statistical analysis: All obtained data were expressed as Mean±SD of at least three experiments and analyzed by One-way Analysis of Variance (ANOVA) test with the aid of GraphPad Prism (version 9.0.0) Software (San Diego, USA). The $p<0.05$ were regarded as statistically significant.

RESULTS

Cell survival rate: To evaluate cell viability percentage after administering laser, *P. major* and the combination of two treatments, we conducted an MTT analysis. The HFFF2 cell survival rate has been illustrated in Fig. 1, from which we can see that the highest *P. major* concentration that had no

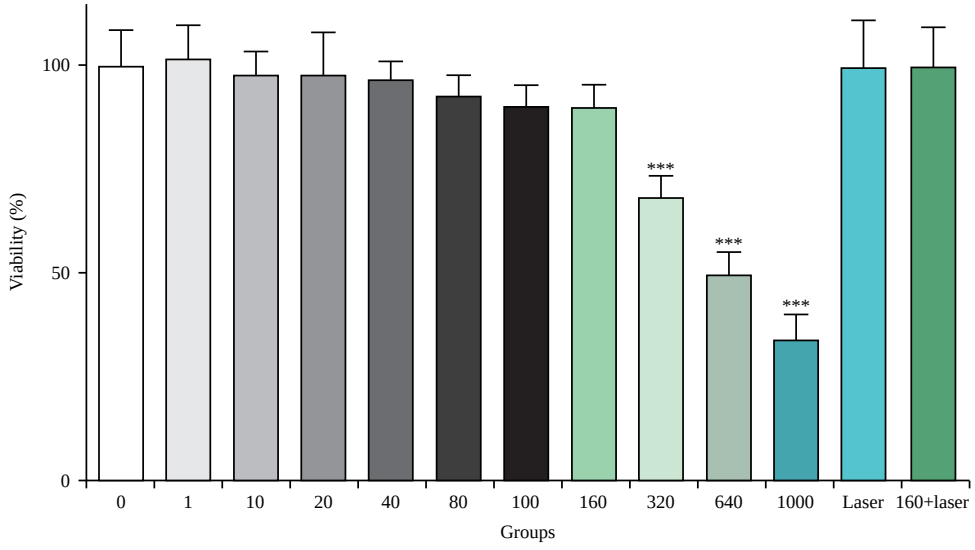

Fig. 1: Cell survival rate, HFFF2 cells after 24 and 48 hrs of treatment with different concentrations of *P. major* and the *P. major*-laser combination relative to untreated control were measured by MTT assay
Data are expressed as Mean±SD of three MTT assay experiments (***$p<0.001$)

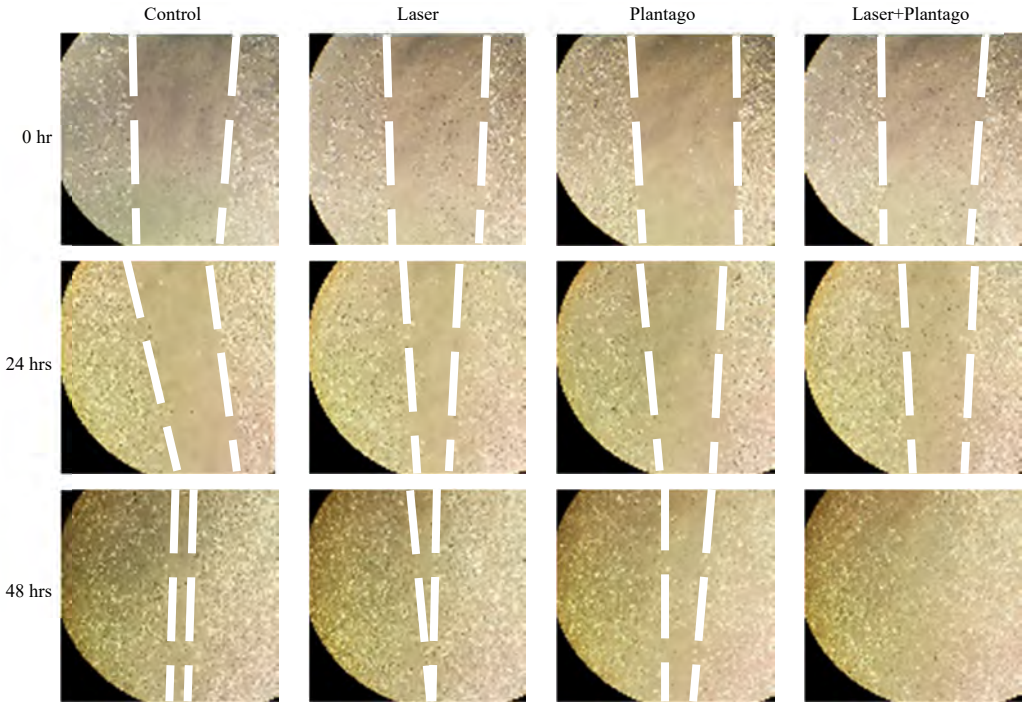

Fig. 2: Wound healing microscopic analysis, microscopic images taken from HFFF2 cells treated with *P. major*, laser and the *P. major*-laser combination were determined through scratch assay

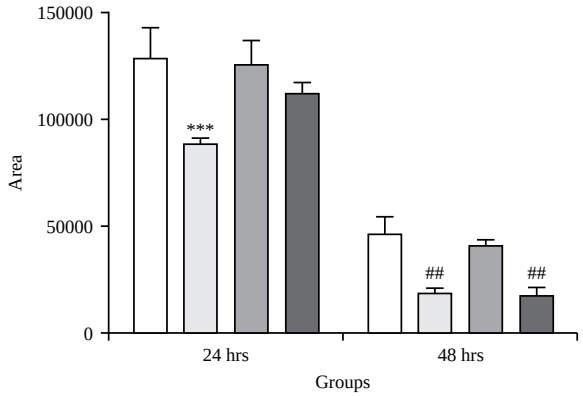

Fig. 3: *In vitro* scratch assay, scratch area changes in HFFF2 cell culture plates after 24 and 48 hrs of treatment with *P. major*, laser and the *P. major*-laser combination relative to untreated control, measured by wound healing scratch assay

Data are expressed as Mean±SD of three replications (*p<0.05, **p<0.01 and ***p<0.001)

cytotoxicity was 160 µg mL^{-1}. According to this finding, we selected 160 µg mL^{-1} *P. major* for further investigations. In addition, Fig. 1 showed that laser treatment alone and in combination with 160 µg mL^{-1} *P. major* had no significant cytotoxic impact on HFFF2 cells as compared to control cells.

Wound healing analysis: Wound healing ability of combined laser and *P. major* was assessed through scratch assay. The microscopic images of cells treated with *P. major*, laser and their combination for 24 and 48 hrs were shown in Fig 2.

The wound healing capacity of laser and *P. major* combination relative to laser and *P. major* treatments alone has been shown in Fig. 3. As it can be seen from this figure, laser treatment for 24 hrs showed the statistically significant reduced scratch area in comparison with other groups. However, 48 hrs of HFFF2 treatment with different groups showed that laser alone and its combination with *P. major* could effectively lead to decreased scratch area, indicating stronger wound healing effect of these treatments.

Gene expression analysis: The real-time PCR technique was applied to measure the expression of inflammatory cytokines under the effect of laser, *P. major* and their combination. The VEGF, TNF-α and IL-6 gene expression after HFFF2 cell treatment with laser, *P. major* and their combination were highlighted in Fig. 4. This figure indicates that the combination of *P. major* and laser could remarkably upregulate mRNA levels of the VEGF ($p<0.01$), TNF-α ($p<0.0001$) and IL-6 ($p<0.0001$) genes.

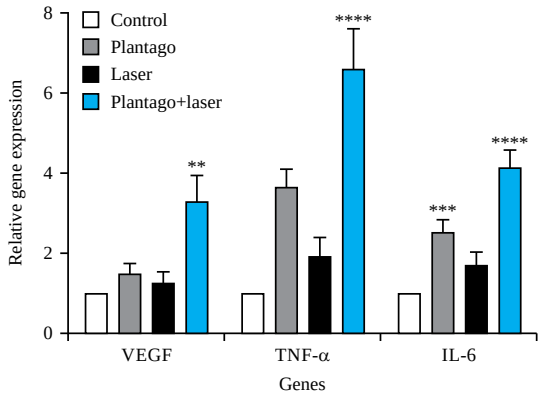

Fig. 4: Gene expression analysis, expression of VEGF, IL-6 and TNF-α after 24 and 48 hrs of treatment with *P. major*, laser and the *P. major*-laser combination relative to untreated control; assessed by real-time PCR

Data are expressed as Mean±SD of three replications (**p<0.01, ***p<0.001 and ****p<0.0001)

DISCUSSION

In recent years, approaches to improve the wound healing process have attracted the attention of many researchers. One of these approaches is a combination of traditional and modern therapeutic options for wound healing[19]. For many years, numerous plant extracts have been used in traditional medicine for the remediation of skin wounds, mainly due to their therapeutic properties such as cell stimulating, anti-inflammatory and antimicrobial activities[20]. The use of *P. major* in wound healing dates back to ancient times. It contains polyphenols, gluten, tannins, flavonoids and coumarins, which possess anti-inflammatory, anti-fever, anti-ulcerative, anti-microbial, anti-diarrhea and wound healing properties[7]. The PDT is a modern therapeutic approach to wound healing that is capable of stimulating acute inflammation, which leads to alternations in physiological processes in wounds and enhances the wound healing process[21]. Thus, we set out this study with the aim of assessing the ability of laser and *P. major* combination to improve the wound healing process.

In this study, results found that laser treatment alone and in combination with *P. major* was not cytotoxic to HFFF2 cells. This finding shows that the administration of laser can lessen the cytotoxicity impact of high concentrations of *P. major*, indicating an effective synergistic effect of laser and *P. major* combination. This synergistic effect of combination therapy is in consistent with a recent study, which reported that the mixture of *P. major* and aloe vera accelerates the process of wound closure by inducing fibroblast proliferation and increasing collagen bundles[22].

Herein, HFFF2 cells treated with the combination of laser and *P. major* showed increased closure of scratch, which may explain the positive wound healing effect of our combined treatment on wound healing. This might be the result of induced fibroblast proliferation and migration, which are key steps in the wound healing process[10,23,24]. These findings also match earlier studies, which demonstrated that the use of laser[25,26] and *P. major*[12,27] leads to the reduced area of the scratch site and improves wound healing.

Another important finding of this study was increased gene expression of VEGF, TNF-α and IL-6 in HFFF2 cells treated with the combination of laser and *P. major*. The VEGF is considered a principal growth factor, being expressed in many cell types that have a contribution to the wound repair process including fibroblasts, endothelial cells, smooth muscle cells, macrophages, platelets and neutrophils[28]. Notably, overexpression of VEGF in fibroblasts accelerates vascularization and improves wound healing[29]. Current finding was in agreement with a very recent study, which demonstrated increased levels of VEGF after administering combination of curcumin and low level laser[30]. Formerly, researchers have shown that both *P. major*[31] and PDT[32] at specific doses and under certain conditions were able to stimulate angiogenesis by upregulating the expression of one of the most important pro-angiogenic factors called VEGF. Hence, we can interpret that our combined *P. major*-laser therapy might have the potential to expedite wound repair by inducing angiogenesis.

As mentioned above, we also observed increased expression of IL-6 when cells were treated with the combination of laser and *P. major*. As a pro-inflammatory cytokine, IL-6 plays a central role in the timely resolution of wound healing[33]. The IL-6 signaling triggers JAK/STAT and MAPK signaling pathways, thereby leading to fibroblast migration to the site of injury, which subsequently produces collagen[34,35]. These mechanisms further support current finding upon increased IL-6 expression after the use of *P. major* and laser combination.

We also observed upregulated TNF-α gene expression in HFFF2 cells treated with the laser and *P. major* combination. When a wound is occurred, neutrophils stimulate gene expression of cytokines such as TNF-α, which plays an early regulatory role in the inflammatory phase of the wound healing and promotes reepithelialization and wound closure[36]. Here, the increased cytokine expression showed that our proposed combination therapy can probably serve as a suitable and effective option to improve wound closure.

CONCLUSION

This research demonstrate the pronounced effect of *P. major*-laser therapy on the HFFF2 proliferation and its benefits to wound healing procedure like inducing the gene expression of VEGF as a predominant growth factor and pro-inflammatory cytokines TNF-α and IL-6.

SIGNIFICANCE STATEMENT

In this study, those data provide strong *in vitro* evidence to support the potential wound healing activity of the laser and *P. major* combination. Nevertheless, more research is needed to determine the efficacy of *in vivo* administration of combined *P. major* and laser in wound repair process.

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