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The effects of Shilajit on periodontal ligament cells in wound healing: a comprehensive in vitro study

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Abstract

Background Shilajit is a pale-brown to blackish-brown fluid that varies in consistency and is released from rock layers in various mountain ranges across the world. For thousands of years, traditional medical systems in several nations have included shilajit in one form or another as a rejuvenator and adaptogen. Numerous medicinal qualities have been attributed to it, several of which have been confirmed by contemporary scientific analysis. This in vitro study was established to investigate the effect of shilajit on human Periodontal ligament **(**hPDL) cell wound closure.

Methods The cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) test following a 24-hour exposure of shilajit. With the use of an inverted phase contrast microscope, morphological alterations were noted. Acridine orange/ethidium bromide dual labeling, to evaluate the apoptotic cell death in shilajit treated cells. An in vitro wound healing test was utilized to evaluate wound healing in wounded hPDL cell monolayers for 24 h in the presence or absence of shilajit. The Matrix metalloproteinases-2 and 9 (MMP-2 and MMP-9) in hPDL cells treated with shilajit were measured using the enzyme-linked immunosorbent assay (ELISA) technique, and real-time PCR was used to examine gene expression linked to wound healing and apoptosis.

Results Shilajit's cytotoxicity evaluation on hPDL cells showed that dosages as high as 3 mg/mL had no adverse effects and maintains the cell viability, suggesting a possible stimulatory effect on cell growth. Cell viability was reduced significantly ($p < 0.05$) by dosages more than 4 mg/mL, indicating cytotoxicity at higher concentrations. According to the scratch wound healing assay, shilajit administration at doses of 2 and 3 mg/mL accelerated wound healing and improved cell migration in hPDL cells. Shilajit promoted a controlled inflammatory response and supported periodontal ligament healing by upregulating the expression of genes involved in collagen synthesis, collagen type I alpha 1 chain (COL1A1) and pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and, interleukin-1-beta (IL-1β), according to real-time PCR data. In addition, Shilajit raised the protein levels of MMP2 and MMP9, two important enzymes involved in tissue remodeling. Shilajit-treated hPDL cells showed a substantial increase of cell proliferation and no discernible apoptotic activity.

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Conclusions Our research offers novel proof that shilajit promotes hPDL cell migration and proliferation, which in turn promotes wound closure. According to these results, Shilajit may improve tissue regeneration, accelerate wound healing, and encourage the growth of periodontal ligament cells.

Keywords Shilajit, Migration, Periodontal ligament cells, Wound closure, Proinflammatory cytokines

Introduction

Periodontitis is a chronic inflammatory disease that is highly common and can cause loss of bone, attachment, and even teeth [1]. The disease being most prevalent, over 90% of adults globally suffer from periodontitis. Periodontal diseases can lead to the progressive destruction of the supporting structures of the teeth, including the gingiva, periodontal ligament, and alveolar bone. This deterioration often results in gingival recession, increased tooth mobility, and, if left untreated, eventual tooth loss [2]. Although necessary for the condition, oral periodontopathogens are insufficient to cause periodontitis. The onset and progression of periodontitis are influenced by additional risk factors, including smoking, genetic predisposition, and certain systemic disorders [1]. Periodontal microbes like Fusobacterium nucleatum can cause the loss of periodontal structures by attaching to specific receptors such as toll-like receptor (TLR) 2 and triggering an inflammatory host response [3]. The traditional therapies for periodontitis, scaling and root planing, successfully prevent the progression of the disease. Yet, regeneration of the damaged periodontal tissues remains a huge concern.

The tooth roots are supported and shielded by the hPDL, which is situated between the alveolar bones and the tooth root. New functional collagen fibers must be inserted into the growing cementum and new bone in order to promote periodontal regeneration. hPDL comprises several cell types, such as fibroblasts, osteoblasts, cementoblasts and some precursor cells. According to [4] these cells are crucial for tooth nutrition, homeostasis, and tissue healing. According to [5] hPDL cells have the ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages. According to earlier research, hPDL cells can develop into osteoblasts through chemical induction and mechanical stress. According to earlier research, hPDL cells can develop into osteoblasts through chemical induction and mechanical stress [6]. Additionally, hPDL cells encouraged the growth of new cementum on the periodontal ligament's renewal.

Natural remedies have been utilized for many dental issues since the dawn of civilization and continue to be a safer option. These substances include antibacterial, anti-inflammatory, antioxidant, analgesic, and antimutagenic qualities that make them useful for both preventing and treating oral conditions like gingivitis, periodontitis, infections, and even cancer. These components are increasingly used in dental care products since they are inexpensive, safe, and biocompatible, especially because around 80% of people globally rely on natural therapies for their medical needs [7, 8]. They are essential for controlling dental diseases such tooth decay and periodontal disease, reducing bacterial biofilms, and improving dental formulations. The nanohydrogel formulation significantly enhances wound healing by promoting human gingival fibroblast cell migration, proliferation, and extracellular matrix remodeling, suggesting its potential for periodontal regeneration [9]. Additionally, their wound-healing, antimicrobial, and anti-inflammatory qualities greatly support oral health, with several natural compounds showing remarkable efficacy in wound-healing applications. Quercetin, for instance, alleviates AGE-induced wound healing impairment in human gingival fibroblasts by reducing inflammation, oxidative stress, and NF-κB activation, suggesting its potential in managing diabetic periodontitis [10].

The kind of cells that repopulate the root dictate periodontal repair. The original periodontal tissue architecture can be restored with the use of regenerative treatment methods which encourage hPDL cell growth, migration, and attachment. However, the results of the regenerative therapeutic techniques that are currently accessible are not always predictable and can be impaired by various circumstances [11, 12]. Therefore, one of the primary objectives in dental research is to find novel compounds with the ability to regenerate. Shilajit, sometimes referred to as shilajit, shilajatu, mumie, or mummiyo, is an exudate with varying consistency that ranges in color from pale brown to blackish brown, originating from rock layers in numerous mountain ranges worldwide, particularly the Himalayan ranges in the Indian subcontinent [13, 14]. In several regions of the world, such as Australia [15], Iran, and the Altai Mountains [16], shilajit is widely used in largely the same chemical composition and has many medicinal benefits in traditional folk medicine.

Shilajit is made of organic molecules (60–80%), inorganic substances (20–80%) and several elements (20–40%). Previous research indicates that the primary chemical components of Shilajit are DBP chromoproteins, fulvic acid, and dibenzo-alpha-pyrones (DBPs). Furthermore, potassium, calcium, and magnesium make up the majority of Shilajit's total mineral composition, with sodium and sulfur falling behind [17]. Shilajit also contains endogenous amino acids including histidine, proline, glycine, tyrosine, arginine, and aspartic acid, as

well as exogenous amino acids like methionine, leucine, and threonine [17]. Shilajit has been shown to possess a variety of biological properties, including antioxidant, antibacterial, anti-inflammatory, and immunomodulatory effects. It has anti-hyperlipidemia, spermatogenesis, anticancer, gastroprotective, and oogenesis properties as well. Fulvic acid and DBPs provide Shilajit its antioxidant properties [18]. Additionally, fulvic acid protects the electrical potential and inhibits cell death and facilitates importation of minerals [19]. Shilajit's safety and therapeutic effectiveness have been both pre-clinically and clinically investigated [18, 20]. The bioactive compounds present in Shilajit have shown therapeutic potential in managing various health conditions, including metabolic, respiratory, neurological, musculoskeletal, and reproductive disorders. Notably, it has been studied for its benefits in conditions such as osteoarthritis, anemia, diabetes, and Alzheimer's disease [21–23].

Shilajit, when administered orally following tibia fracture surgery enhanced bone regeneration, according to an Iranian clinical trial [24]. Nevertheless, studies on shilajit's direct impact on hPDL cells' capacity for wound healing are lacking. Even with the advancements in traditional periodontal therapies, there is still a need for treatments that improve the regenerative potential of periodontal tissues in addition to treating symptoms. Restoring periodontal health and function depends on effective wound healing within the hPDL. Shilajit is a fascinating option for investigation in the context of periodontal wound healing due to its abundant biological activities. It is critical to comprehend Shilajit's potential to facilitate hPDL cell wound healing in order to create novel periodontal regenerative medicine treatment approaches. This research aims to close the current knowledge gap by thoroughly investigating Shilajit's effects on hPDL cells' capacity for wound healing. Our goal is to clarify the underlying mechanisms governing Shilajit-induced migration of hPDL cells through an array of in vitro tests.

Materials and methods

This study was conducted according to the guidelines of the Declaration of Helsinki. The Research Ethics Committee, College of Dentistry, King Khalid University has reviewed and approved the research with a reference no: IRB/KKUCOD/ETH/2023-24/011. All methods were performed in accordance with the relevant guidelines/regulations/legislation. Commercially available 200 mg Himalayan Shilajit paste (Lajit, USA) was purchased and used in the current study. Informed consent to participate in the study was obtained from the participants.

Preparation of the Shilajit solution

Using commercially available 200 mg Himalayan Shilajit paste (Lajit, USA), a Shilajit solution was made, the Shilajit paste was dissolved in 10 ml of Dulbecco's Modified Eagle Medium (DMEM) media and centrifuged for 10 min (500 x g) at room temperature. Following a syringe filter (0.22 µm - Merck Millipore, USA) filtering of the supernatant, 10 mg/ml of the stock solution was further diluted in medium to produce specific concentrations (0.5, 1, 2, 3, 4 and 5 mg/ml) [25].

Cell culture

This work made use of the hPDL cell culture experimental model. Cells were isolated from healthy human teeth extracted for clinical reasons from a 34-year-old male patient, following informed consent and ethical approval. Forceps were used to hold the coronal section of the tooth, the hPDL was scraped using a #15 scalpel blade. After that, the tissue was placed on sterile Petri dishes and washed with DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL), and fungizone (5 mg/mL) at 37 °C in a humidified environment with 5% CO2. After that, it was divided into smaller pieces and transferred into tissue culture flasks. These tissue fragments were kept in the supplemented DMEM and allowed to adhere to the flask's surface in a 37 °C environment with 5% CO2. Culture medium renewal was carried out twice a week until the cells reached confluence. After that, the cells were subcultivated and detached from the culture flasks using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid for three to five minutes. The first and second passages of cells were used to create a master bank and a working bank of cells, respectively. These cells were then suspended in a cryopreservation medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO) and kept in liquid nitrogen until they were multiplied to the point where they could be used for experiments.

The cell viability (MTT) assay

An MTT assay was performed to assess the hPDL cells' vitality. This colorimetric technique, known as the MTT assay, quantifies the decrease in yellow MTT caused by mitochondrial succinate dehydrogenase in living cells. In a CO_2 incubator set up at 37˚C with 5% CO2, individual human periodontal ligament cells were grown in 96-well plates at a concentration of 5×10^3 cells/well using DMEM media containing 1X Antibiotic Solution and 10% fetal bovine serum (Gibco). Following a wash with 100 µL of 1X PBS, the cells were treated with Shilajit (0.5–5 mg/ml) that had been collected from the previous procedure. The cells were then incubated for 24 h at 37˚C with 5% CO2. At the completion of the treatment period, the media was aspirated from the cells. After adding

0.5 mg/mL MTT prepared in plain media, the mixture incubated in CO_2 incubator for 4 h at 37˚C. After entering the cells' mitochondria, the MTT turns into an insoluble formazan crystal with a deep purple hue. Following the incubation period, the cells' media containing MTT was removed, and 100 µl of PBS was used to wash the cells before solubilizing them with DMSO, an organic solvent. Thus, a microplate reader at 570 nm is used to measure the liberated, solubilized formazan product. The following formula was used to calculate the cell viability percentage: cell viability = [O.D of treated cells/O.D of control cells] × 100.

Morphology study

We determined the ideal doses (IC-50: 2 and 3 mg/ml) for additional research based on the MTT experiment. Cell morphological changes were analyzed using an inverted phase contrast microscope. Six-well plates seeded with 2×10^5 cells, and were treated with Shilajit (2 and 3 mg/ml) for 24 h for hPDL cells. The media was removed and the cells were once rinsed with phosphate buffer saline (PBS pH 7.4) at the end of the incubation period. Under an inverted phase contrast microscope, the plates were observed.

Detection of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The effects of Shilajit on hPDL cell death were also assessed using the previously mentioned AO/EtBr dual staining method. Following a 24-hour treatment with Shilajit (2 and 3 mg/ml), the cells were cleaned with ice-cold PBS. AO (0.1 mg/mL) and EtBr (0.1 mg/mL) were added to the cells for incubation. The apoptotic modifications of the stained cells were then examined with a fluorescence microscope.

Table 1 Primer sequences

Gene	Forward Primer	Reverse Primer	Tm
TNF-a	GCCCAGGCAGTCAGATCATCT	TTGAGGGTTTGCTACAACATGG	60
COX-2	CCGGGTACAATCGCACTTAT	GGCGCTCAGCCATACAG	58
IL-1b	TGGCAATGAGGATGACTTGTTC	CTGTAGTGGTGGTCGGAGATT	60
COL1A1	GACATGTTCAGCTTTGTGGACCT	GCCAGCAGATCGGTTCCAG	60
PCNA	TTGCTCTTGAGGCTTGTGC	GTAGTTGGGGCCTGTATGGT	60
p53	AGGCCTTGGAACTCAAGGAT	TGAGTCAGGCCCTTCTGTCT	60
Caspase-3	GCTATTGTAGGCGGTTGT	TGTTTCCCTGAGGTTTGC	55
Bcl2	CATGTGTGTGGAGAGCGTCAAC	CAGATAGGCACCCAGGGTGAT	58
GAPDH	CGACCACTTTGTCAAGCTCA	CCCCTCTTCAAGGGGTCTAC	58

Scratch wound healing assay

As in our earlier research, a well-known in vitro wound healing model was used to assess the in vitro wound healing. In brief, six-well culture plates were seeded with 2×10^5 human hPDL cells per well. After creating a wound on the cell monolayer with a 200 µl tip, it was cleaned with PBS and photographed under an inverted microscope. Following a 24-hour treatment session with Shilajit (2 and 3 mg/ml) and control cells receiving serum-free culture media, the wounded area was captured on camera using the same microscope. Furthermore, for each treatment group, the tests were conducted thrice.

MMP-2 and MMP-9 quantification with ELISA

Human periodontal ligament cells were seeded in 6-well plates in triplicate at a density of 2×10^5 cells/well and cultivated in specified medium until about 80% confluence. After treating the cells with Shilajit (2 and 3 mg/ml) for 24 h, the Human Matrix metalloproteinase 2 and 9 ELISA Kit (Abbkine, USA) was used as per manufacturer's instructions, to measure the MMP-2 and MMP-9 activity of Shilajit-treated hPDL cells.

Real time PCR

Real-time PCR was used to analyze the gene expression of apoptotic genes and wound healing biomarker genes. Following Shilajit treatment (2 and 3 mg/ml), total RNA was extracted utilizing Trizol Reagent (Sigma) as per standardized protocol. Using a PrimeScript, first strand cDNA synthesis kit (TakaRa, Japan) and 2 µg of RNA reverse transcription-based cDNA was synthesized. Specific primers were used to amplify the targeted genes (Table 1). GoTaq® qPCR Master Mix (Promega), which includes all of the PCR components and SYBR green dye, was used to perform the PCR reaction. Utilizing a Biorad CFX96 PCR system, real-time PCR was carried out. The comparative CT approach was utilized to analyze the results, and Schmittgen and Livak's 2−ΔΔCT method was employed to calculate the fold change.

Statistical analysis

Three separate experiments were carried out, and the mean or median was taken into consideration as the final measurement value based on the distribution. One-way analysis of variance (ANOVA) with Tukey's post hoc test followed by The Student's t-test evaluated the variations between the test and control groups. Group differences were deemed significant if the probability "p" value was equal to or less than 0.05 ($p \leq 0.05$). The Statistical Package of Social Sciences (SPSS 25.0) program (IBM, Armonk, NY, USA) was used to conduct all statistical tests.

Results

Cytotoxicity assessment

The survival rate of hPDL cells in the presence of various concentrations (0.5–5 mg/mL) of Shilajit was assessed at three distinct time points in order to determine the proper dosage. The results of the MTT experiment on human hPDL cells exposed to varied shilajit doses are shown in Fig. 1. The mean cell viability at 1 mg/mL and 2 mg/mL concentrations was 103.530 ± 4.926 and it was 104.166 ± 5.148 and 96.206 ± 6.605 at 24-hour time point. The same type of results was obtained at 48 and 72 h (Fig. 1). With percentages above 100%, a notable increase in cell viability was seen, demonstrating that there is a potential stimulating effect on cell proliferation. 3 mg/ml concentrations which are not statistically significant. At 4 mg/ml and 5 mg/ml, the cell viability percentage dropped compared to the control group (81.699 ± 2.525 and 74.368 ± 4.857, respectively). Above 4 mg/ml, there was a statistically significant ($p < 0.05$) difference in cell viability between the treated group and the control group (Fig. 1). Shilajit supplementation up to 3 mg/mL was utilized for in vitro studies as the MTT test revealed no harmful impact from this dose. Considering the inverted microscope photos, morphological alterations in hPDL cells treated with shilajit appear to have occurred only at 4 mg/ml doses as compared to the control group. In particular, compared to the control group, the cells treated with shilajit at doses of 2 and 3 mg/ml exhibited a more elongated and spindle-like appearance. These alterations in cell shape imply that the shilajit might be affecting the cells' cytoskeleton, which is involved in wound healing and cell migration (Fig. 2).

Effect of Shilajit on hPDL cells wound healing potential

The impact of shilajit on hPDL cell monolayer wound closure was examined. Following a 24-hour incubation period with shilajit, the wound closure of injured hPDL cell monolayers was monitored. Wounded monolayers that had not been treated with shilajit served as the control. Shilajit nanogel treatment appears to have accelerated wound healing and cell migration in comparison to the control group, according to the in vitro scratch wound healing experiment (Fig. 3). In particular, the assay images demonstrated that, at 24 h, the scratch wounds of treatment groups closed more quickly than those of the control group. Both the 2 mg/ml and 3 mg/ml shilajit concentrations showed this effect. These results suggest that the shilajit may have the potential for promoting wound healing and cell migration in human periodontal ligament cells (Fig. 3).

Treatment with Shilajit promotes wound healing process through modulating the inflammatory signaling molecules

The delicate process of wound healing is facilitated by the interplay of multiple biological pathways, with gene expression playing a critical role in orchestrating these processes. The inflammatory and reparative processes in the periodontal ligament are mostly regulated by inflammatory cytokines. One such gene, the tumor necrosis factor-alpha (TNF-α) gene, has been thoroughly investigated in relation to wound healing. Early in the inflammatory stage of wound healing, TNF-α is released. It facilitates the recruitment of immune cells to the site of injury, aiding in the removal of any pathogens and debris. TNF-α also has a role in activating many signaling pathways that control cell migration and proliferation.

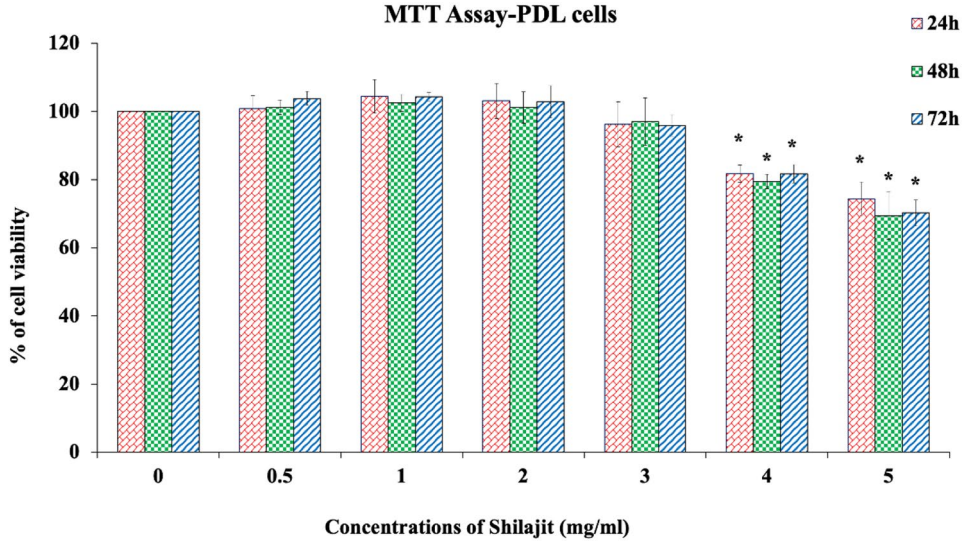

Fig. 1 The cytotoxic effects of Shilajit (0.5 to 5 mg/ml) on hPDL cells. Cells were treated for 24 h, 48 h and 72 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD ($n = 3$). * compared with the control blank group, $p < 0.05$

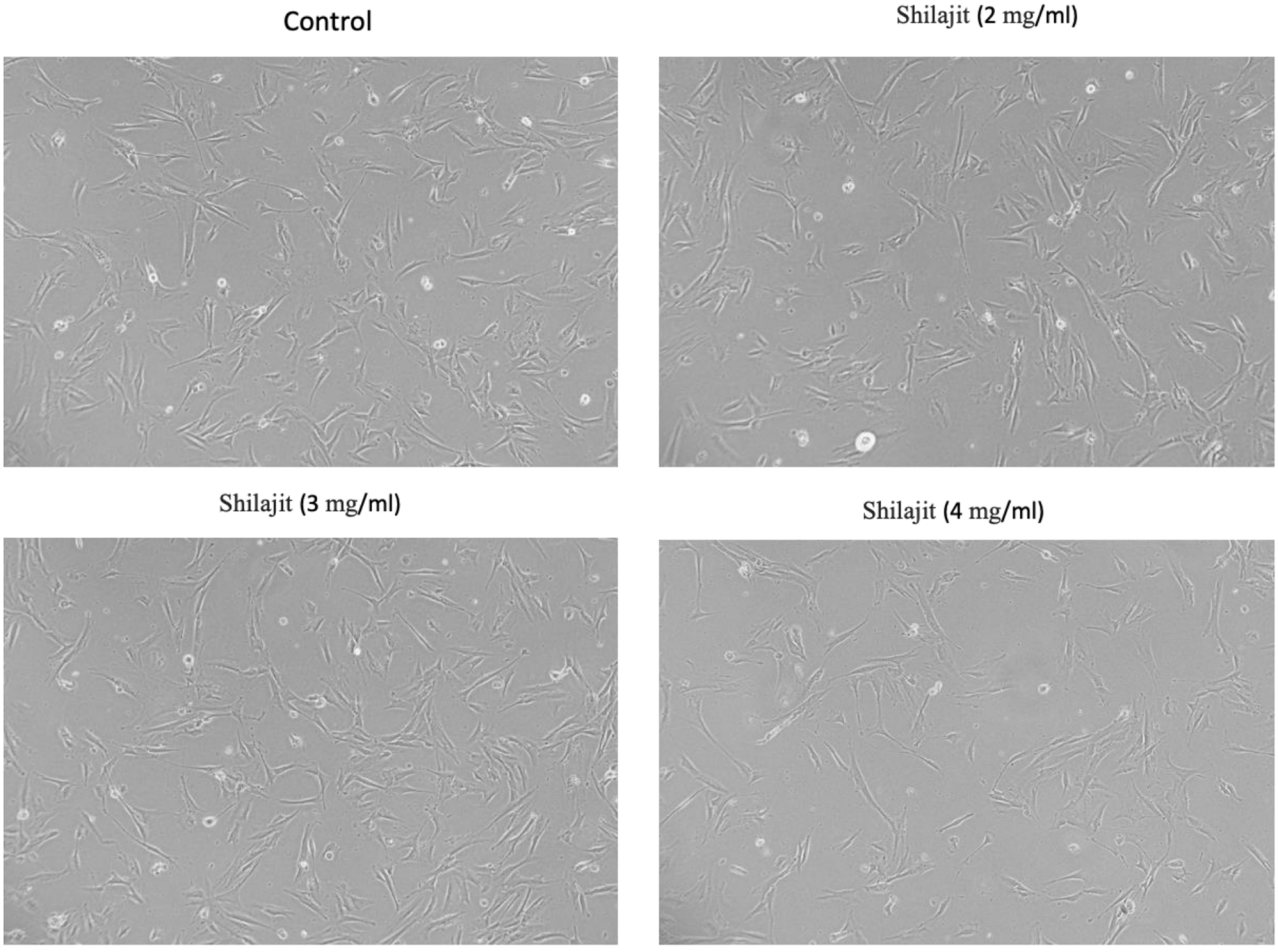

Fig. 2 Effect of Shilajit on cell morphology of human hPDL cells. Cells were treated with Shilajit (2, 3 and 4 mg/ml) for 24 h along with the control group. Images were obtained using an inverted phase contrast microscope

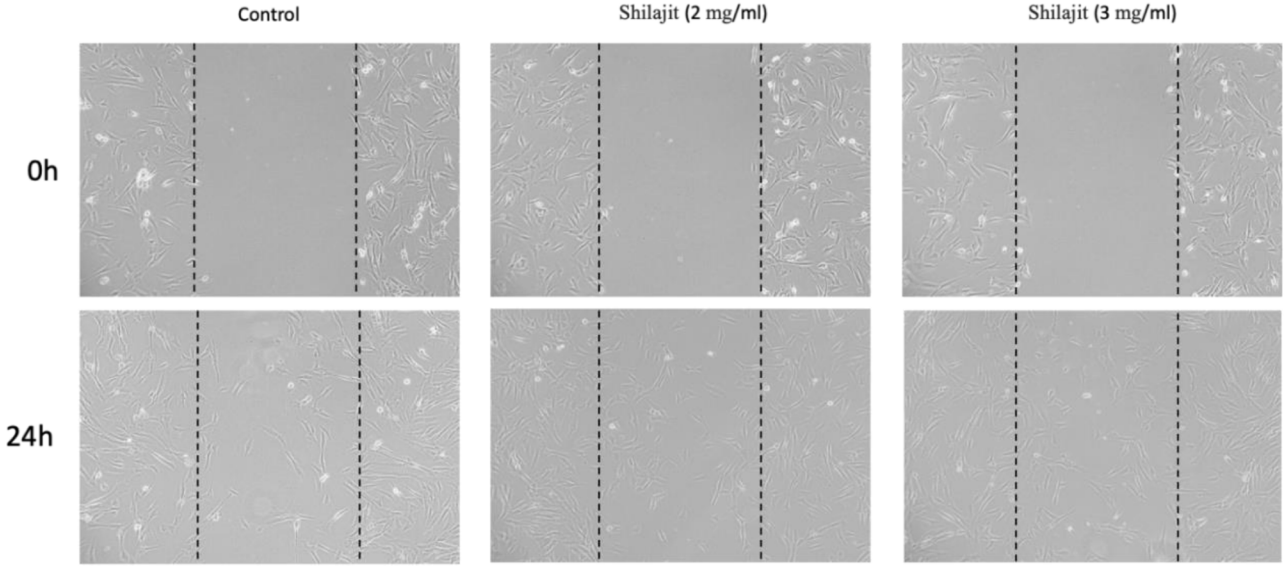

Fig. 3 In vitro scratch wound healing assay. Human hPDL cells were injured and cell migration assay with and without treatment of Shilajit (2 and 3 mg/ml) was performed for 24 h. Images were obtained using an inverted Phase contrast microscope

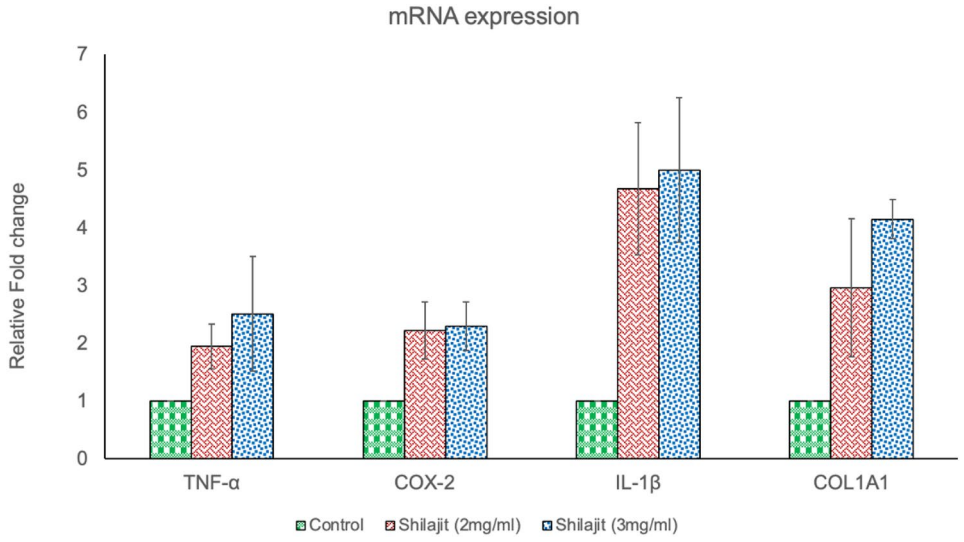

Fig. 4 Effect of Shilajit (2 and 3 mg /ml) on TNF-a, COX-2, IL-1B and COL1A1 gene expression in hPDL cells. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus drug treatment groups at $p < 0.05$ level

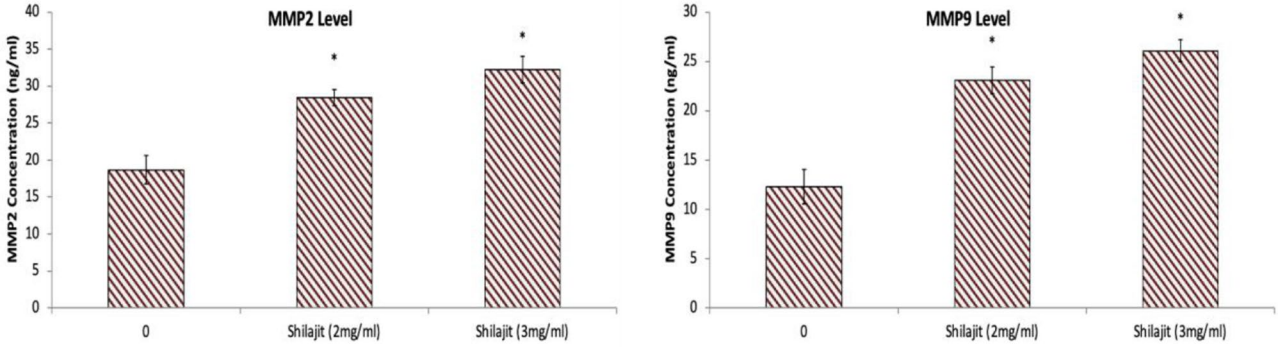

Fig. 5 MMP2 and MMP9 levels in hPDL cells were assessed by ELISA kit. The condition media was used from control and Shilajit 2 and 3 mg/ml treated cells. Each bar represents the mean ± SD. *Statistically significant difference between two groups ($p < 0.05$); #statistically significant difference between control and treated ($p < 0.05$)

It seems that shilajit influences the expression of the TNF-α, IL-1β, Cox-2, and COL1A1 genes to promote wound healing. Shilajit contributes to a more controlled inflammatory environment by upregulating the expression of pro-inflammatory cytokines like TNF-α and IL-1β, according to Real Time PCR results. In addition, shilajit has demonstrated the ability to control the expression of the inflammatory enzyme Cox-2 gene, which may impact the inflammatory response in periodontal ligament cells. Additionally, shilajit increases hPDL cells' expression of the COL1A1 gene, which is essential for the periodontal ligament's structural integrity and healing. Our results establish shilajit as a promising candidate for further study in the context of periodontal health and wound healing in the oral cavity, as it may positively influence the control of inflammatory and reparative processes in periodontal ligament cells (Fig. 4).

Effects of Shilajit on MMP2 and MMP9 protein level in HhPDL cells

Two enzymes that are involved in tissue remodeling, including the degradation of extracellular matrix components, are Matrix Metalloproteinase 2 (MMP2) and Matrix Metalloproteinase 9 (MMP9). The control of MMP2 and MMP9 expression is essential for preserving tissue homeostasis and is frequently linked to inflammatory and wound-healing processes. Shilajit may have an effect on MMP expression, which could influence the balance between tissue breakdown and rebuilding. Shilajit's regulation of MMP2 and MMP9 in hPDL cells may have an effect on the periodontal ligament's extracellular matrix turnover. In order to maintain tissue integrity, MMP activity must be properly regulated. In this study, shilajit-treated hPDL cells showed a substantial amplification of MMP2 and MMP9 protein levels (Fig. 5).

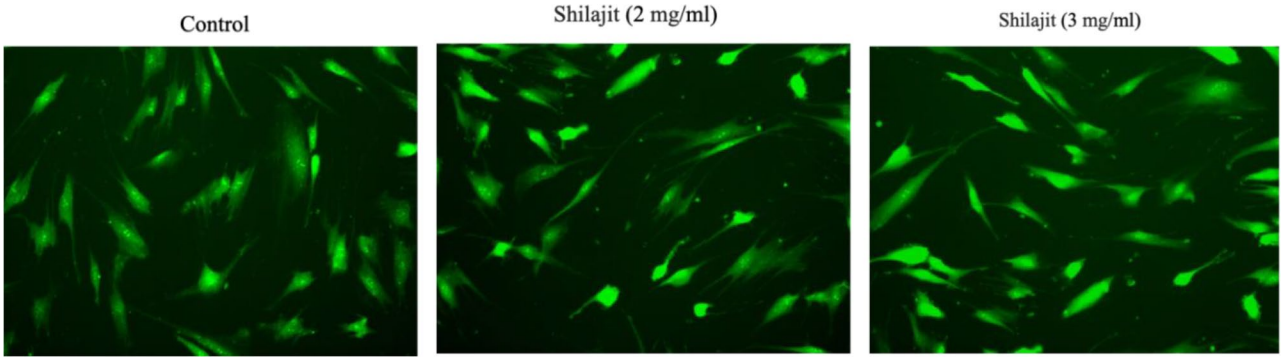

Fig. 6 Human hPDL cells were treated with Shilajit (2 and 3 mg/ml) for 24 h along with the control group. After the treatment, the cells were incubated with AO/EtBr dual staining. Images were obtained using an Inverted Fluorescence Phase contrast microscope

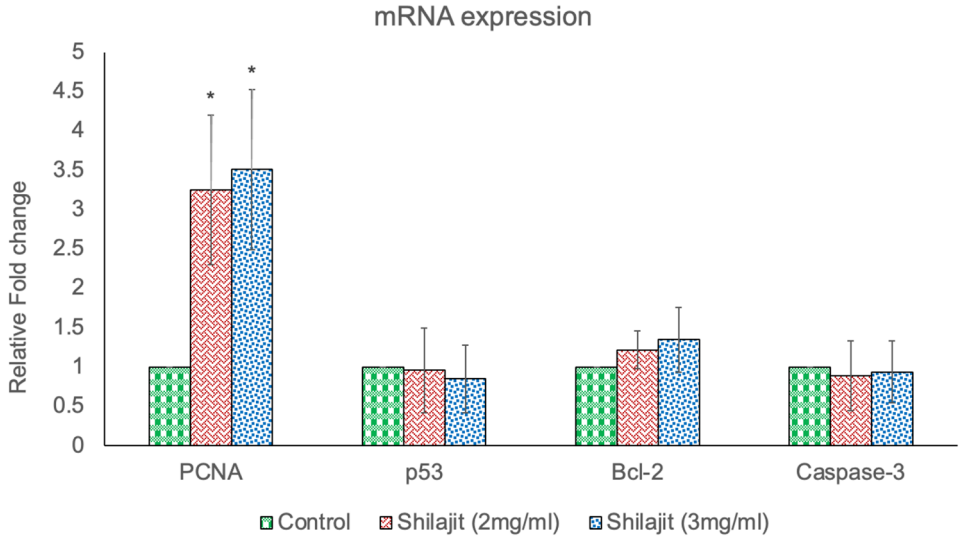

Fig. 7 Effect of Shilajit (2 and 3 mg /ml) on Proliferative marker gene (PCNA) and Apoptotic genes (p53, Bcl-2, Caspase-3) expression in hPDL cells. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus drug treatment groups at $p < 0.05$ level

Effects of Shilajit on proliferation and apoptosis

We further studied the effects of shilajit on the functioning of these cells since wound closure also depends on cell number, which is decided by apoptosis and proliferation. Following a 24-hour incubation period with shilajit (2 and 3 mg/ml), we observed that hPDL cells had no apoptotic nuclei. Figure 6 illustrates the different features of the cells after they were exposed to the AO/EtBr dye. The nuclei of healthy and live cells are normal, and the nucleus has a stunning reticular pattern of green stain. One essential dye that can be used to stain both living and dead cells is orange. Only cells with damaged or ruptured membranes are stained by ethidium bromide. Live cells are invariably green in color. Early apoptotic cells are green in color and contain brilliant green spots in their nuclei due to chromatin condensation and nuclear fragmentation. In contrast to necrotic cells, late apoptotic cells color orange because they also consume ethidium bromide, but their nuclei are condensed and occasionally fragmented. However, in the current investigation, shilajit therapy did not result in any of these apoptotic features in hPDL cells over a 24-hour treatment period (Fig. 6). Shilajit treatment of hPDL cells led to a marked upregulation of PCNA expression, a specific marker for cell proliferation (Fig. 7). However, there was no change seen in the expression of the apoptotic marker Caspase-3, Bcl-2, or p53 between the groups. The absence of alterations in the expression of p53, Bcl-2, and Caspase-3 genes in periodontal ligament (hPDL) cells treated with shilajit implies that shilajit may not have a substantial effect on these particular genes linked to cellular survival and apoptosis within the experimental setup.

Discussion

The present investigation looked into the way shilajit affected hPDL cell wound closure. Our research is the first to offer a unique demonstration that shilajit promotes hPDL migration and, consequently, wound

closure. This suggests that shilajit may be beneficial for periodontal remodeling and healing. Furthermore, the hPDL cell wound closure is accelerated by the activation of inflammatory cytokines. According to Mukaddes Yerebakan demonstrated that pterostilbene (PTS) promotes wound healing by reducing inflammatory markers such as TNF-α and IL-6, which in turn enhancedantioxidant activity [26]. Ayurvedic medicine has traditionally employed shilajit, a resinous exudate rich in minerals, fulvic acid, and bioactive chemicals, for its supposed health advantages [27]. Recent studies have further highlighted the biological relevance, emphasizing its medicinal potential. For instance, Rajpoot et al. show that Shilajit alleviates testicular toxicity caused by chemotherapy drugs, indicating that it plays a protective role in the dynamics of testicular germ cells and the regulation of steroidogenesis [28]. Similarly, Hussain et al. explore the usage of Shilajit coatings, revealing their ability to enhance the properties of hydrogels and demonstrating how they can be used in biomedical materials [29]. Perumal et al. concentrate on the environmentally friendly manufacturing of Shilajit's zinc oxide nanoparticles, which show encouraging anticancer activity, indicating its potential for cancer treatment [30]. Together, these results highlight the significance of shilajit in Ayurvedic medicine. Its broad-spectrum bioactivity is being investigated for a number of therapeutic uses, including as antioxidative, anti-inflammatory, and anticancer properties. Its potential for wound healing has drawn attention recently. Cell culture models are frequently used in in-vitro research to evaluate cellular responses related to migration, proliferation, and expression of molecular markers associated with tissue repair in wound healing studies examining the effects of shilajit.

Previous studies have shown that in comparison to 2D cultures with or without OM, Shilajit under 3D conditions increases osteoblast development of Alg-encapsulated ASCs. Shilajit enhanced the osteogenic effect of OM in addition to exhibiting osteoinductive qualities. These results are consistent with what earlier research on Shilajit has shown. Shilajit can inhibit osteoclastogenesis while stimulating MSCs to differentiate into osteoblasts and enhancing mineralization, according to in vitro data [31, 32]. When compared to placebo groups, systemic injection of Shilajit accelerated bone growth in individuals with tibial and femoral fractures [24, 32]. Shilajit may increase the pace of bone formation through the production of polysaccharides, nucleic acids, and the majority of proteins and hormones required for the growth of cells and tissues, according to in vivo experiments conducted in a rat osteoporotic model [33]. Furthermore, consistent with our results, osteoblast proliferation was boosted by Shilajit when given to MG63 cells, osteoblast-like cells, in vitro [34]. Consequently, Shilajit demonstrated a favorable impact on hPDL cell proliferation based on the in vitro findings of our investigation. Even at high concentrations of up to 3 mg/ml, shilajit did not adversely influence cell viability, according to in vitro experiments.

The objective of this in vitro investigation was to see how a shilajit impacted the viability of human periodontal ligament cells. After 24 h of treatment with varied concentrations of shilajit (0.5–5 mg/ml), the vitality of cultured hPDL cells was assessed. The cellular viability was determined using the MTT test. The difference in cell viability index is insignificant, according to the statistical analysis. Hence, the shilajit (upto 3 mg/ml) is cytocompatible with human hPDL cells, according to a cytotoxicity test. Furthermore, there were no apparent alterations in cell morphology after receiving Shilajit treatment. This suggests that the shilajit may be at the 3 mg/ml concentration and cytotoxic at these higher doses 4 mg/ml and above.

Here, for the first time, we show that Shilajit induces cell migration potential of hPDL cells, which was assessed by scratch wound healing assay. Previous research by Das et al. [35] reported that Shilajit taken orally stimulates blood vessel growth and endothelial cell migration, which can impact skin health and rejuvenation. Similarly, research has demonstrated that Shilajit has positive benefits on the mouse model's wound-healing process. According to our results, Shilajit not only had cytotoxic effects on hPDL cells but also marginally increased cell proliferation. For this reason, we looked into the possibility of a higher dosage of Shilajit to improve wound healing while maintaining the best level of survival. Additionally, we conducted a scratch test in the present study to look into the impact of Shilajit on hPDL migration. Our findings supported earlier research and shown that, while treated shilajit preserved spindle-shaped morphology, it stimulates cell migration and proliferation to facilitate the healing of scratch areas [35].

The process of wound healing involves a complex interplay of various biological mechanisms, and the expression of genes plays a crucial role in orchestrating these intricate processes. One such gene that has been extensively studied in the context of wound healing is the tumor necrosis factor-alpha (TNF-α) gene and its downstream molecules [36]. TNF-α is released early in the inflammatory phase of wound healing. Simultaneously, shilajit has shown the potential to modulate Cox-2, an enzyme involved in inflammation, potentially influencing the inflammatory response in periodontal ligament cells [37]. Moreover, shilajit may play a role in enhancing COL1A1, which is crucial for the structural integrity and repair of the periodontal ligament [38]. In our real time PCR results showed that shilajit treatment significantly increased the TNF-α, IL-1β, Cox-2, and COL1A1 gene expression in hPDL cells.

Our research on shilajit suggests its potential role in promoting the wound healing process in periodontal ligament cells through the modulation of key molecular pathways. Shilajit appears to exert its effects by influencing TNF-α, IL-1β, Cox-2, and COL1A1. These findings suggest that shilajit may have a positive impact on the regulation of inflammatory and reparative processes in periodontal ligament cells, making it a subject of interest for further investigation in the context of periodontal health and wound healing within the oral cavity. Furthermore, we analyzed the impact of shilajit treatment on MMP-2 and MMP-9 protein level in hPDL cells. The regulation of MMP2 and MMP9 expression is crucial in maintaining tissue homeostasis and is often associated with processes such as wound healing and inflammation [39, 40]. Specific research on the effects of shilajit on MMP2 and MMP9 expression in human periodontal ligament (hhPDL) cells may be limited. Our ELISA data demonstrated a substantial rise in MMP-2 and MMP-9 expression following shilajit administration in hPDL cells.

Furthermore, in this investigation, impact of shilajit treatment on cell proliferation and apoptosis were analyzed. The AO/EtBr dual staining approach was used to identify apoptotic cells in hPDL cells following treatment with shilajit. There were no cells that displayed signs of cell death, such as apoptosis or necrosis, according to the findings. This indicated that the majority of cells were in a stable state. As a result, treating shilajit would give cells with more favorable conditions. Earlier studies on fibroblast and osteoblast cells proves that the shilajit is osteo compatible and highly osteogenic in vitro. Further, we assessed mRNA expression of proliferative marker PCNA and apoptosis marker genes p53, Bcl-2 and caspase-3 in shilajit treated hPDL cells. And the results showed that PCNA expression was significantly increased and there are no significant changes in the apoptosis gene expression in shilajit treated hPDL cells. Moreover, our experiments showed that shilajit also increased the speed of hPDL cell migration. Since both proliferation and migration determine the wound fill rate, our observation that shilajit promotes wound healing is supported by these findings. Therefore, in the present study the shilajit may be potentially used in wound healing and soft tissue engineering applications. Nonetheless, the limitation of any in vitro model, including this one, is that it cannot fully mimic the plethora of actions and interactions of different cell and tissue types that take place in vivo. However, further investigations are required in animal models and clinical trials in order to validate their potential as wound-healing agents. Albeit, these findings unveiled an unexplored avenue in regenerative medicine, offering promising insights into the synergistic potential of these components in enhancing wound closure and tissue regeneration.

Conclusion

In conclusion, our comprehensive study of shilajit's impact on human periodontal ligament cell wound closure has produced some very interesting findings. The data obtained during every phase of our investigation firmly substantiate the claim that shilajit acts as a trigger for the migration and growth of human peripheral blood cells. These results highlight shilajit's possible therapeutic potential in the field of periodontal health. The enhancement of cellular migration and proliferation that has been seen suggests that shilajit may be a crucial component in enhancing the complex processes involved in periodontal tissue repair. Its function in promoting periodontal healing is becoming progressively evident as the intricacies of this natural substance are being discovered out, opening up new research directions and possible clinical applications.

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Author contributions

Conceptualization, AA, JH and MIK; Data curation, JH and EP; Formal analysis, RMM, AWA and AM; Investigation, JH and EP; Supervision, JH, and MIK; Validation, AWA and AM; Visualization, RMM and EP; Writing – original draft, AA, RMM, AWA, AA and MIK; Writing – review & editing, JH, EP and MIK. All authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was conducted according to the guidelines of the Declaration of Helsinki. The Research Ethics Committee, College of Dentistry, King Khalid University has reviewed and approved the research with a reference no: IRB/KKUCOD/ETH/2023-24/011. All methods were performed in accordance with the relevant guidelines/regulations/legislation. Commercially available 200 mg Himalayan Shilajit paste (Lajit, USA) was purchased and used in the current study. Informed consent to participate in the study was obtained from the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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