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In Vitro Evaluation of *Achillea Millefolium* on the Production and Stimulation of Human Skin Fibroblast Cells (HFS-PI-16)

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ABSTRACT

Aim: In the present study, we aimed the effects of the hydroalcoholic extract of *Achillea millefolium* (HEAML) on human skin fibroblast cells (HSF-PI-16) proliferation, stimulation and growth properties. **Methods:** Initially, using HSF-PI-16 monolayer culture, we created one line scratch method as an in vitro wound closure and after 3 days monitored via an inverted microscopy. **Results:** HEAML selectively inhibited proliferation of HSF-PI-16 cells at higher concentration (>20.0 mg/mL), and stimulated at lower concentrations (<20.0 mg/mL). Following, HSF-PI-16 media treatments up to 72 h, HEAML demonstrated significantly elevated proliferation rates (p<0.05) and stimulation in a scratch wound assay (p<0.04). Furthermore, the morphological analysis of HSF-PI-16 cells at culture media were detected the figures of round to spindle, non-adherent, immature and mature cells. **Conclusion:** These results clearly demonstrate the absence of any toxic effect of HEAML on human skin fibroblasts. To the best of our knowledge, this is the first report elucidating potential mechanisms of action of HEAML on fibroblasts proliferation, and stimulation, offering a greater insight and a better understanding of its effect in future studies.

Key words: Fibroblasts, Proliferation, ***Achillea millefolium*****, Skin, Culture.**

1. INTRODUCTION

Nowadays, medicinal plants are widely utilized around the world as an alternative to pharmaceutical drugs. Although herbal products are considered to have fewer adverse effects compared with synthetic drugs, they are not completely free from side effects or toxicity (1). Recent phytochemical, pharmacological and clinical studies on various species have confirmed their ethno-medicinal properties. *Achillea millefolium* L. (Asteraceae) is widely used in Europe as a herbal remedy for the treatment of spasms, such as digestive complaints, as an emmenagogue, and for irregular menses (2). Several *A. millefolium* constituents including monoterpenes, sesquiterpenes, flavonoids and caffeoylquinic acid derivatives have been reported (3). Each class of phytoconstituents can at least in part account for specific medicinal properties of *A. millefolium*. Leaves and flowers from *A.millefolium* have been used for centuries for anti-inflammatory actions, such as rheumatism, skin inflammation and allergic rhinitis, wound healing and amelioration of diaphoresis and high blood pressure (4, 5, 6). Plant cell and tissue culture is a useful technology for producing plant specific bioactive compounds, and many hairy root cultures of it, which present genetic and biochemical stabilities similar to those of the mother plant, are established for more stable and efficient production of their active constituents (7).

Fibroblasts play a potential function in proliferation and migration in response to chemotactic, mitogenic and modulatory cytokines, and also autocrine and paracrine interactions (8, 9), and these cells have many applications in tissue engineering, genetics and aging research, diagnosis of peroxisomal disorders, cell nuclear transfer, and cell reprogramming (8, 9, 10). Following severe damage, fibroblasts infiltrate the wound, secrete growth factors and

cytokines, and deposit fibrotic extracellular matrix factors such as collagen I and fibronectin (11). Fibroproliferative is a term used to describe myofibroblast-rich tissues (12, 13, 14, 15). Proliferation of myofibroblasts and myofibroblast precursors is important in fibroproliferative tissues, therefore understanding myofibroblast proliferation and differentiation is important in normal tissue remodeling and in fibroproliferative pathologies, such as Dupuytren's contracture (12), hypertrophic scars (16), myocardial infarction fibrosis (17), and pulmonary fibrosis (18), among others. The aim of study was to evaluate scratch wound assay on proliferation and cell migration of HSF-PI-16 cell number in RPMI + %10 FBS culture media after 24,28,and 72h at control and HEAML groups

2. MATERIALS AND METHOD

Aerial parts from *Achillea millefolium* L. were collected was collected in May 2014, from populations growing in Ilam province, Iran. The plant was identified in Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Science, Mashhad, Iran. A voucher specimen was deposited in the herbarium of the above mentioned college.

Aerial parts (leaves, stalks and stems) of *A. millefolium* L. were air-dried in an oven at 35 to 45 C for 5 days. *A. millefolium* leaves were used in this study since these parts are utilized in traditional medicine. The aforementioned parts were ground to a fine powder. One gram of powder was extracted using 10 mL of ethanol/distilled water solution (alcohol/water = 8:2, v/v), with centrifugation at 3000 r/min for 15 minutes, and then the supernatant was collected. This process was repeated three times. Solvents were then removed by evaporation (19, 20). The dry plant was cut and pulverized. Three concentrations of hydroalcoholic extracts (AEAM; 2.5%, 5% and 10%) were prepared using the powdered plant material. For this, 100 g of powder was placed in 500 mL of distilled boiling water and kept at room temperature for 15 min. The infusions were freshly prepared just prior to administration at dosages of 0, 1000 or 2000 μg/kg of AEAM. The extraction, fractionation and chemical characterization of the hydroalcoholic (HEAM) and dichloromethane subfractions (DCM-2) were performed as previously described (21).

Normal human skin fibroblasts were obtained from Science Cell Research Laboratories (Carlsbad, CA, USA) and were cultured in Dulbecco's modified Eagle's medium (DME, Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with fetal bovine serum (FBS) to a final concentration of 10% (Gibco, Burlington, ON, Canada). The medium was changed three times a week. When the cultures reached 90% confluence, the cells were detached from the flasks with a 0.05% trypsin-0.1% ethylene diamin tetra acetic acid (EDTA) solution, washed twice, and resuspended in FBS-supplemented DME medium. In each experiment, the fibroblasts were used between passages 4 and 5.

Human skin fibroblast (HSF-PI-16) were seeded uniformly (5.0×10^4 cells/well) into 6-well macroplates (with each well containing a single cover-slip) at a RPMI + %10 FBS environment , after which time a scratch (wound) was created on each confluent monolayer using a 1mL sterile pipette tip (PipetTipFinder, A division of Lab Procurement Services, LLC, Knoxville, TN, USA) perpendicular to the bottom of the dish, and then the plates incubated for a further 24 hr at 37°C/5% $CO_2$and grown for 24 hr with conditioned media being replenished every ~6 h. A wound scratch was made across the centre of each confluent cell culture using a sterile 1mL pipette tip and any non-adherent cells were washed off with 1× PBS. Conditioned media was then reapplied to the cells according to the previous treatment regimen and the plates incubated for a further 24 hr at 37°C/5% CO_2. Cellular fixation was then achieved using 4% formaldehyde/PBS (Sigma-Aldrich, UK) applied for 30 min at RT, followed by permeabilization with PBS/0.1% Triton X-100 solution (Sigma-Aldrich, UK). The cells were then treated with 1:1000 diluted IC50 extract, then at next step one concentration of IC50 hydroalcoholic extract was added than the previous plates. The fibroblast cells at plates were then incubated at 37°C for 12 hr with the conditioned medium. To record scratch wound closure, images were captured at 16, 20,38,44,64,70 and 88 hr time points in the same position using an Olympus IX71 microscope (Olympus, Southborough, MA, USA).Each well was photographed six times using objective inverted microscopy (Olympus, UK) and the number of cells that migrated into the scratched area counted (using a standardized scratch area for each image) as described previously (22).

Kinetic analysis of the migration process of HSF was measured using time-lapse microscopy. Cells were seeded in 2 compartments separated by a silicon insert (Ibidi devices). At subconfluence, inserts were removed, cells were rinsed twice with PBS to remove non-adherent cells, and the culture medium was replaced by fresh medium containing or *hydroalcoholic extract* of *Achillea millefolium* leaves (HEAML) (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, a, 12.5 ,and 25 mg/mL) and supplemented with 0.1% SVF. Cell migration was followed using an inverted time-lapse microscope equipped with an environmental chamber at 37°C under 5% CO_2. The microscope was controlled by Metamorph software. Images were taken every 30 min and recorded with a charged-coupled camera for 48 h. Cell migration was quantified using Image J software.

All data is presented as mean +/− standard error of the mean (SEM) from repeated experiments. Statistical analysis was performed by using Student's t-test, $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. HEAML produced no side effect on the human skin fibroblasts

We expanded human skin fibroblasts in culture and, as described above, transfected the cells with HSF-PI-16 levels (Figures 1 and 2). Similar to mesenchymal cells, over 30 % of the transfected fibroblasts expressed IC50, and also, we assumed that these HEAML might contribute to the transformation of the mesenchymal HSF-PI-16 cells into mentioned plates, and after determining the optimal dose and schedule, we added mentioned concentrations of HEAML at 24, 48 and 72h of culture and harvested the cells for analysis at days 1, 2 and 3. Under these conditions, over 20 and /or 30% of the cells expressed IC50 at high

Time	Control	0/19	0/39	0/78	1/56	3/12	6/25	12/5	25
24h	0/338	0/001	0	-0/001	0/002	0/008	0/011	0/073	0/181
	0/387	0/002	0/003	0/003	0/008	0/011	0/022	0/155	0/278
	0/37	0/002	-0/002	-0/002	0/002	0/008	0/017	0/136	0/247
48h	0/304	0/007	0/004	0/005	0/006	0/016	0/021	0/016	0/351
	0/309	0/004	0/004	0/004	0/011	0/025	0/03	0/208	0/401
	0/312	0/002	0/002	0	0/004	0/014	0/023	0/117	0/311
72h	0/324	0/007	0/003	0/004	0/002	0/008	0/006	0/005	0/152
	0/357	0/005	0/003	0/004	0/004	0/006	0/006	0/006	0/233
	0/22	0/007	0/003	0/005	0/007	0/008	0/015	0/012	0/306

Table1. Scratch wound assay was performed on proliferation and cell migration of HSF-PI-16 cell number in RPMI + %10 FBS culture media after 24,28,and 72h at control and HEAML groups

time, as compared with control group when the cells were exposed to HSF-PI-16 (Figures 3, 4 and 5). The HSF-PI-16 cells also expressed different levels of time and dose. HSF-PI-16 cells treated with HEAML became round to spindle and small, as confirmed by morphological and side- and forward-scattered culture analysis (Figures 1 and 2). The cells became non-adherent, and acquired cell surface markers and morphological features typical of immature and mature cells of all hematopoietic lineages. These data are supported by the cell viability analysis. As shown in Figures 3, 4 and 5, difference in cell density was observed between the HEAML -exposed and non-exposed fibroblast cultures. There was also difference when cells were exposed for 24, 48 and 72 h to either low or high mg/ml. It is, however, interesting to note that the exposure to HEAML for 3days produced a significantly ($p<0.05$) higher cell count compared to the non-exposed control culture. These results clearly demonstrate the absence of any toxic effect of HEAML on human skin fibroblasts.

3.2. HEAML promoted cell migration of HSF-PI-16 and scratch closure and morphological observation

In this study, fibroblast migration capacity in the presence or absence of HEAML was investigated using an *in vitro* wound scratch assay. Migration of HSF-PI-16 into a scratch wound inflicted across confluent cultures (following 81 of media treatments) was significantly elevated in HEAML media-treated fibroblasts compared to non-HEAML control media ($p<0.05$). Fibroblasts revealed cultures were confluent prior to scratching and HSF-PI-16 cells of HEAML groups orientated in parallel monolayers in contrast to control cells, which formed whirl-like aggregates in a similar manner to the whirl-like nodular structures demonstrated in post-confluent cultures. Therefore, HSF-PI-16 cells treated with HEAML media elicited strong migration into the scratch wound. Furthermore, HSF-PI-16 media treatments of HEAML elicited statistically significant ($p<0.05$) increased proliferation after 24, 48 and 72hrs compared to the HSF-PI-16 media controls (Figures 3, 4 and 5). After 72 h significant increases were maintained (Fig 5). After 48 h and 72 h, HSF-

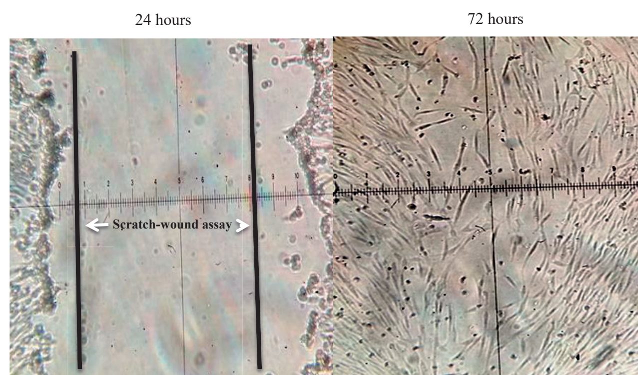

Figure 1. Microscopic observation of HSF-PI-16 fibroblasts. A representative image is shown of primary cultured HSF-PI-16 fibroblasts (400× magnification). The cells appeared elongated or flattened, and some were triangular.HSF-PI-16 fibroblasts closed scratch assay wounds more rapidly in control and treat groups. a) The extent of wound closure in scratch assays of HSF-PI-16 fibroblasts at 72 h in cultures with 10% FBS. Data shown are the means (± SEM), *p < 0.05. b) FBS in serum supplemented cultures; the HSF-PI-16 fibroblasts migrated into the "wound area" more rapidly in cultures. Representative images are shown of the progression of wound closure at 24 and 72 h, at which time the HSF-PI-16 fibroblasts can be seen to fill the wound area almost to the exclusion of HEAML. Original magnification ×100 and 400.

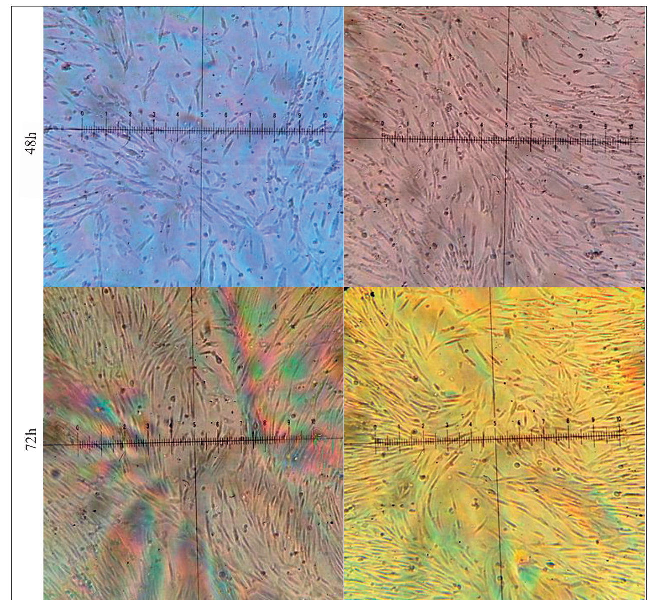

Figure 2. Fibroblasts in cultures. 5.0×104 fibroblasts, were cultured in 6-well macroplates (with each well containing a single cover-slip) for 72 hours, and then HEAML, were seeded into the wells with HSF-PI-16 cells. On day 2, fibroblasts tended to cover the entire bottom of the well and on day 3, the overgrowing fibroblasts resulted in some cells grew on others. In the presence of IC50, wound closure was nearly complete at this time (72h). Original magnification ×400. b) There was a significant increase in the extent of wound closure in HSF-PI-16 scratch assays in HEAML compared with the control medium at 27 h. Data shown are means ± SEM, *p < 0.05. Representative inverted microscope images of HSF-PI-16 scratch assays immediately after the scratches had been made and then after 24 h in the presence of extract free IC50 versus test medium.

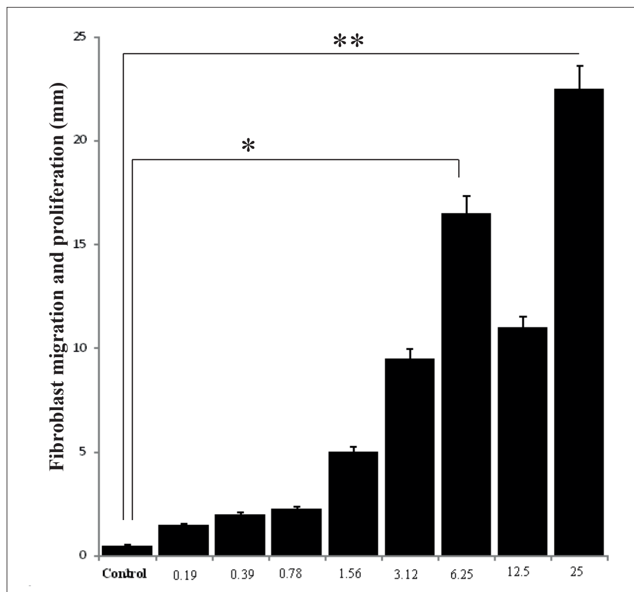

Figure 3. Proliferation and cell migration in scratch wound invasion assay after 1day (24h) of conditioned media treatment. Significantly increased (*p<0.05) proliferation and cellular viability was observed in HSF-PI-16 treated with HEAML media versus respective control media after 24 h. As shown, there was a significant increase in HSF-PI-16 cell number in RPMI + %10 FBS culture media after 24 h, which was beyond the time course for wound closure by HSF-PI-16 cell type in the presence of HEAML

PI-16 media elicited consistently higher proliferation than media controls. Nevertheless, this difference was statistically significant. Similar trends were observed in human skin fibroblasts. RPMI + %10 FBS cultures of HSF-PI-16 showed statistically (p<0.05) elevated proliferation when treated with IC50 media versus corresponding HSF-PI-16 control media (Table 1). Overall proliferation rates were greater, and statistically significant, in HEAML versus the corresponding HSF-PI-16 cells media regimens. As cell

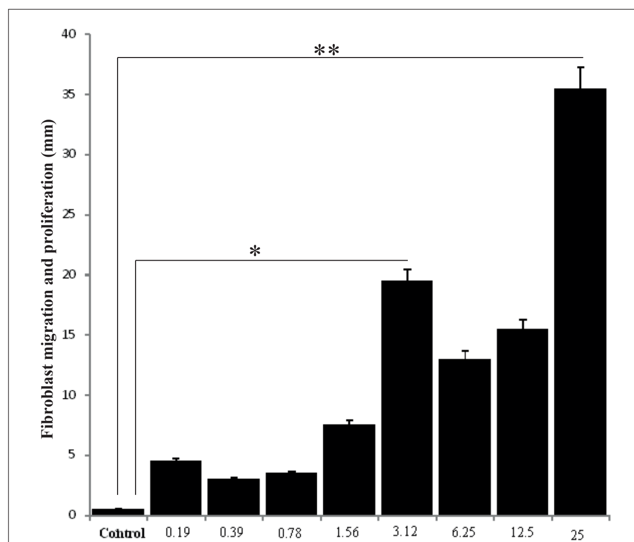

Figure 4. Proliferation and cell migration in scratch wound invasion assay after 2day (48h) of conditioned media treatment. Significantly increased (*p<0.05) proliferation and cellular viability was observed in HSF-PI-16 treated with HEAML media versus respective control media after 48 h. As shown, there was a significant increase in HSF-PI-16 cell number in RPMI + %10 FBS culture media after 48 h, which was beyond the time course for wound closure by HSF-PI-16 cell type in the presence of HEAML

proliferation, growth factor production and cell contractile capacity were promoted by HEAML, we hypothesized that HEAML could promote cell growth and that this stimulatory effect could be transmitted from one cell generation to another. To address this possibility, fibroblasts were exposed to HEAML and subsequently cultured for 24 h. They were then trypsinized and subcultured for 24, 48, and 72 h to measure cell growth.

3.3. Evaluation of HSF-PI-16 index by inverted microscope

Cell index (CI) measured by the label free inverted microscope system is used as a guide to monitor cellular behavior including spreading, proliferation, viability and morphology (Table 1) (Figures 1 and 2). Dynamic responses in our panel of cells (to different conditioned media) were continuously monitored following 24 h cell synchronization. HSF-PI-16 cells treated with HEAML conditioned media produced steady increases in CI from 24–72 h, indicating an enhanced HSF-PI-16 cell spreading, proliferation and changes in cellular morphology .The highest overall CI was from HSF-PI-16 followed by HEAML media treatments in which treated media elicited consistently higher CI for the duration of 72 h. The addition of HEAML in the culture medium did influence the wound closure and therefore had significant effect on the migratory capacity of fibroblasts in culture. However, a slight increase, approximately 2.4% of the wound closure, was observed in the presence of HEAML (25μmg/ml) after 72 h of incubation, and this increase was significant (Figure 5). We also observed that the fibroblasts cultured with the HEAML at RPMI + %10 FBS media had a higher density than those cultured alone. On day 3 after the cultures were started, the fibroblasts overgrew and formed several layers of cells when grown alone. To warp up, to understand the long-term effects of IC50 or HEAML and culture on the proliferation of fibroblasts, some cultures were observed on the 1, 2, and 3 day after the cultures were initiated. As shown in Figure 5, the fibroblasts in the

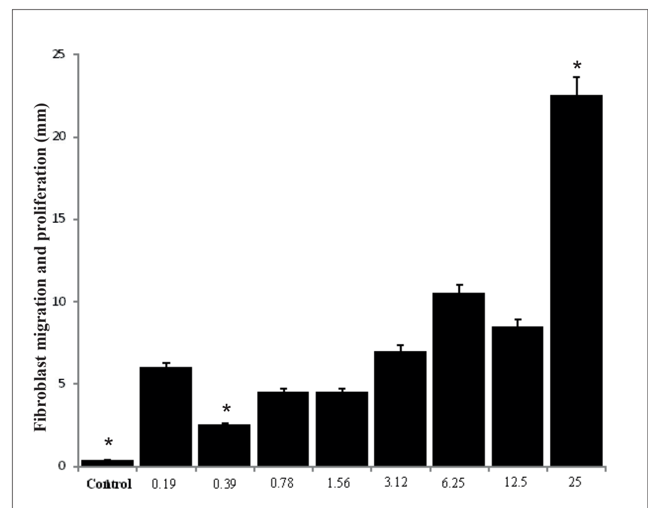

Figure 5. Proliferation and cell migration in scratch wound invasion assay after 3day (72h) of conditioned media treatment. Significantly increased (*p<0.05) proliferation and cellular viability was observed in HSF-PI-16 treated with HEAML media versus respective control media after 72 h. As shown, there was a significant increase in HSF-PI-16 cell number in RPMI + %10 FBS culture media after 72 h, which was beyond the time course for wound closure by HSF-PI-16 cell type in the presence of HEAML

co-culture with additional IC50 maintained fast proliferation after day 3, while those in other cultures grew much slower. The fibroblasts in the control group exhibited a marked reduction in the proliferation rate following day 3. Since the fibroblasts in the culture with HEAML did not exhibit a reduction in the proliferation rate, the faster fibroblasts proliferation in the treat group during the late stage of the co-culture could be caused by an insufficient level of basic nutrition in media.

4. DISCUSSION

This is the first published *in vitro* study demonstrating the effects of HEAML on fibroblasts proliferation obtained from different anatomical cultures. It is also the first study to compare such effects against the long-standing commercially available controlled. In this study, we used an *in vitro* scratch wound healing model and were able to assess the effects of HEAML compared to control on cultured fibroblasts. Application of *A. millefolium* stimulates cellular spreading, attachment and proliferation in a dose-dependent and time-dependent manner at the cellular level and also shows dose-dependent stimulation at the transcriptional level for many ECM components such as HSF and its growth factors.

Our findings allowed highlighting of an increase in the anabolic pathway of ECM, in particular through stimulation of HSF-PI-16 syntheses. However, it is important to note that our results also indicated an increase in the activity and expression of HSF in the presence of *A. millefolium* extracts. The increased expression in the ECM levels of HSF-PI-16 is correlated to the enhancement of fibroblast cells activity after 48 h and 72 h incubation in the presence of HEAML, and its stimulation should lead to an increase in the amount of ECM components such as HSF. Moreover, our results revealed that *A. millefolium* extract stimulated expression in the HSF-PI-16 levels of ECM. This HEAML is a transcriptional stimulator of ECM genes like those encoding type fibroblast cells activity and is largely responsible for the increase in the expression of fibroblast synthesis occurring during aging of the dermal fibroblasts (23, 24). Indeed, even if our extracts increased the synthesis of HSF-PI-16 *in vitro*, they also stimulated the synthesis of fibroblasts. It appears that the anabolic/catabolic balance could be in favor of matrix material synthesis despite HSF-PI-16 activity stimulation. On the other hand, our extracts increased the mRNA levels of HSF.

Cell proliferation and migration are hallmarks of cell division. DNA duplication is the key step in cell division and it is controlled by different stages: G1/0, G2/M, and S phases of cell cycle (25). Fibroblast cell proliferation and migration phenomena are principally governed by Rho family GTPase like Rac-1, Rho-A, and Cdc-42 (26). The cell cycle phases are coordinated by the expression and/or activation of regulatory proteins, like cyclins (e.g., cyclin A, D, and E), cyclin-dependent kinases (Cdk) mainly Cdk-1 and -2, and Cdk inhibitors. Both cyclins and cyclin-dependent kinases have also been implicated in the formation of actin ytoskeleton in mammalian fibroblast cells (27). These cells, key players in the wound healing process, are responsible for cell-mediated matrix contraction (28, 29). Through a contraction of their actin cytoskeleton, myofibroblasts at the wound site are able to reduce the initial size of the wound, thereby contributing to tissue repair.

A dose-dependent increase in cell number was noticed during 48 h treatment period with maximum and significant ($p<0.05$) increase at 25 mg/mL concentration level, therefore, high concentration of HEAML (25 mg/mL) stimulated cell proliferation and displayed non-cytotoxic effect on HSF-PI-16 cells. Treatment with mentioned dose of HEAML exhibited over 35% increase in cell number at 48 h. However, at other concentration, there was difference in cell proliferation between the control groups cells and cells treated with HEAML. This study supports several other investigations that reported that natural antioxidants increased the number of oral fibroblast (30) and endothelial cells (31) and modulated the growth of endometrial stromal cells (32). Functional end point of fibroblast cell division and proliferation is cell migration (33) into wounded area (30). An established in vitro scratch assay model was used to quantitatively define human skin fibroblast migration in a monolayer cell model by using invert microscope Image J software analysis, as Figure 2 shown, HEAML dose dependently increased the rate of migration into chamber wounded area up to the concentration of 3.12 to 25 mg/mL. The rate of cell migration was similar to that observed in the presence of *A. millefolium* leaves, a potent cell migration inducer. Therefore it is proposed that HEAML is non-cytotoxic with proproliferative capacity to induce fibroblast cell migration, which these processes cultured at a RPMI + %10 FBS media involved with bovine serum for growth. In parallel, a recent study by Smith et al. (34) reported that culture of murine MSC with HSF, where each cell type was separated in a modified Boyden chamber in the presence of bovine and equine serum, enhanced dermal fibroblast migration and proliferation. The response of the dermal fibroblasts included altered expression of genes involved in cell-matrix interactions. Therefore ,this suggests that increased migration of these cells at a FBS media , as stimulated to increased proliferation, was the main factor in the enhanced rate of scratch wound closure in each chamber of HSF.

We routinely observed a higher proportion of fibroblasts with small to large size cells and low granularity in control samples. HSF-PI-16 are for long known to produce clones with heterogeneous characteristics in low density cultures (35, 36). In this context, it is interesting to note that, in long-term cultures of fibroblasts used as an *in vitro* model of cell aging, an increase of the growth potential (37, 38), an increased number of HSF cells (39), and an increased protein production (40), have been reported, which is in agreement with our results. In agreement with mentioned studies, our study showed that constant HEAML at 3.12 to 25 mg/mL at 48 and 72h mediated by promoted skin fibroblast growth, enhanced cell migration, and increased related growth factors production. It is also indicated that such modified cellular behaviors were inherited by the cells up to 2 to 3 days in culture.

Our overall findings thus suggest the beneficial effect of HEAML on wound healing processes. On the other hand, The HEAML-enhanced fibroblast migration and extra-

cellular matrix contraction observed in our study can be explained by the high expression of HSF-PI-16 by the skin fibroblasts following exposure to HEAML at 6.25 and 25 mg/ml. It is important to note the potential dose effect of IC50, as the highest level of HSF-PI-16 expression was obtained at the highest HEAML intensity (25mg/ml) and for the longest exposure period (48and 72 h exposure to IC50).

5. CONCLUSION

We have adapted an established *in vitro* model of skin wound healing; this was with a view to investigating the potential use of HEAML for HSF-PI-16 therapies of cutaneous wounds. The study has demonstrated that HSF-PI-16 brings about wound closure in scratch assays under serum and serum free conditions, where HSF-PI-16 pioneered the wound healing process. These studies provide a novel model system for the *in vitro* study of cell synthesized soluble factors that may influence skin wound healing and provide insight into the potential mechanisms whereby HEAML transplantation is therapeutic. To warp up, the reaction of *in vitro* fibroblasts plays a significant role in the healing process. Hence, the incorporation of HSF in this culture model may be more representative of *in vivo* skin wound healing for the use of this cell type. Moreover,this study clearly demonstrates that HEAML had no cytotoxic effect on human skin fibroblasts seeded on HSF-PI-16 conductive FBS cultures. The fibroblasts non-adhered well and proliferated exponentially following various exposure periods to IC50. Although cells were found to adhere to both the HEAML -exposed and non-exposed FBS cultures, the HEAML -exposed cultures produced a higher number of viable cells and comparable control groups activity, and/ or compared to the non-exposed Medias. Of particular interest is that cell viability/density increased with longer exposure periods (48 and 77 h).

CONFLICT OF INTEREST: NONE DECLARED.

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