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Enhanced Cutaneous Wound Healing by the Leaf Extract of *Achillea eriophora* D.C. Using the *In Vitro* Scratch Assay

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Abstract

Achillea eriophora D.C. (Asteraceae), an endemic species in Iran, is used extensively in Iranian folk medicine to treat various ailments, including gastrointestinal disorders, skin inflammations and wound healing. The present study was aimed to investigate the effects of the methanolic extract of *A. eriophora* leaves on the stimulation of human fibroblast cell proliferation and migration as two pivotal stages in wound healing process. Methanolic extract was prepared by maceration method. Total phenol and flavonoid contents were measured using spectrophotometry. Cytotoxic and proliferation effects of the extract were evaluated by MTT assay on human fibroblast cells. Moreover, migration of the treated cells was assessed by the closure of a denuded area, made by scratching on the confluent monolayer cultures. The results of cytotoxicity assay indicated that the methanolic extract did not have any cytotoxic effect on fibroblast cells when used at concentrations up to 2 µg/mL. Human fibroblast proliferation was stimulated by low concentrations of the extract (0.1-0.8 µg/mL), and the highest level of proliferation was observed in the lowest treatment (0.1 µg/mL). Migration of the cells was induced by intermediate concentrations (1-30 µg/mL) of the extract and the most effective dose was estimated at 1 µg/mL. Due to the fact that lower concentration of the extract, which showed the best proliferation and migration stimulatory effects, were not toxic on the human fibroblast cells, the crude methanolic extract from the leaves of *Achillea eriophora* could be recommended as a potential source for improving the wound healing activity in skin.

Keywords: *Achillea eriophora*; Wound healing; Fibroblast cells; Proliferation; Migration.

Introduction

Achillea eriophora D.C. (Asteraceae) is an endemic species in Iran. This species, with the local name of Sarzardu, is widely used as a folk remedy treatment for many ailments. Traditionally, in Iran as well as many

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European countries it was prevalented the usage of *Achillea* tea for remedy of fever, cold, menstrual cramps, kidney problems and gastrointestinal disturbances. Also this plant has been used as salve for relief of wound and skin inflammation [1, 2].

Several studies have indicated interesting biological activities, including antimicrobial, antioxidant, anti-inflammatory, antispasmodic, antidiabetic, anticancer, antiulcer effects [3] and wound healing activity [4, 5] for different species of genus *Achillea*. Some researchers have attributed antimicrobial and antioxidant properties of *Achillea* species to the substantial amounts of flavonoids and terpenoids present in their extracts or essential oils [6, 7].

Wound healing is a dynamic and interactive process involving soluble mediators, blood cells, extracellular matrix, and mesenchymal cells. It involves four different phases of hemostasis, inflammation, granulation, and tissue remodeling (or maturation), that overlap in time. Fibroblast migration to and proliferation within the wounded area are prerequisites for wound granulation. Fibroblasts then participate in construction of the scar tissue and its remodeling [8].

The aim of the present study was to investigate the effect of the leaf methanolic extract of *A. eriophora* on the proliferation of human fibroblast and also their migrative capacity in a cultured monolayer serving as *in vitro* wound model.

Materials and Methods

Plant material collection and extraction

Floral branches of *A. eriophora* D. C. were collected from pastures of Birjand (N36.34020, E40.710158; 1591 m above sea level), Southern Khorasan province, Iran, in May 2012. The voucher specimen (no. 30348 and 22004) was deposited in Herbarium of Ferdowsi University of Mashhad (FUM), Iran.

Grinded dried leaves of plants were extracted with methanol (1:20 W/V) using maceration method. The extract was filtered through the regular filter paper and evaporated under vacuum, to become powder [9]. For cellular studies, 2 mg from the extracted residue was dissolved in 50 µL dimethyl sulfoxide (DMSO) and diluted in 1950 µL Dulbecco's Modified Eagle's Medium (DMEM) for preparation of a 1000 µg/mL stock solution. Various concentrations of the extract were made by the stock solution.

Total phenolic and flavonoid assay

The total phenolics of the methanolic extract was determined spectrophotometrically at 760 nm according to the Folin-Ciocalteu method [10] and its concentration in different samples was calculated using calibration equation (y=0.0012x+0.0086, r^2=0.966) and expressed as mg of gallic acid equivalent (GAE) per g dry weight.

Total flavonoid content was determined by the aluminium colorimetric method [11] and calibration curve was prepared by different concentrations of quercetin (y=0.0091x-0.0206, r^2=0.995). Accordingly, the results were expressed as quercetin equivalent (mg QE/g dry weight).

MTT assay for cell viability

The MTT assay is widely used to quantify cell proliferation and cytotoxicity. In this study, the MTT assay was used to assess the cell viability and cell proliferation stimulation by the *Achillea* extract. This method allows quick screening of a large number of samples at different concentrations.

For this assay, cells were seeded at a density of 8000 cells/well in 96-well plates in DMEM, supplemented with 10% Fetal Bovine Serum (FBS). After 24 h of incubation, the cells were treated with different concentrations of the extract. Following 24, 48, and 72 h of treatments, the culture media were aspirated and the cells were washed by Phosphate-Buffered Saline (PBS). Then 20 µL of the MTT solution (0.5 mg/mL) was added to each well. After 4 h of incubation, the media were removed and purple crystals were dissolved in DMSO. Absorbance in each well was measured at 450 nm using ELISA reader (AWARENESS, USA) [12].

Cell proliferation assay

The method of Mosmann (1983) was used in order to cell proliferation assay [13]. Human fibroblast cells were seeded in 96-well plate at densities of 8000 cells/well. After 24 h, the medium was changed to basal one (with 1% FBS) and left for another 48 h. Cells were then treated with various concentrations (0.1 to 51.2 µg/mL) of the methanolic extract, diluted in basal medium. Cell growth rates were estimated by MTT assay at different time intervals.

In vitro wound assay

In vitro wounds were induced by a modified protocol of described by Phan et al. (2001), and the scratch assay was performed on cells to study the effect of *A. eriophora* extract on cell migration [14]. Human fibroblast cells were seeded in 24-well plates at densities of 5×10^4 cells/well in the growth medium. After reaching near 100% confluency, the cells were switched to the basal media for 24 h. Then the cells, grown as a monolayer, were scratched using a sterile micropipetting tip. A glass slide was placed across the top of the dish to facilitate steadying of the tip, whilst

scratching, and to enable a straight scratch to be made. The tip was drawn firmly across the diameter of the dish. Cells were washed by PBS in order to remove the loosened debris. A range of concentrations (1-40 µg/mL) of plant extracts were added to a set of 4 wells for each dose. The cells were treated with equivalent amount of DMSO, as control.

For migration assay, cultures were rinsed twice with PBS, fixed by absolute methanol, stained by Giemsa, and examined by a light microscope, equipped with a calibrated ocular, at a magnification of 40 x. Images were taken immediately after wounding followed by further imaging at 24, 48 and, 72 h time points. Cell migration rates were quantified by measuring the change in the wound area (pixels), using Image J software.

Statistical Analysis

Each experiment was performed at least three times. Data were analyzed by one-way ANOVA. Also Duncan test was performed as post hoc analysis. Differences at the 95% level were considered to be significant. Normality, homogeneity of variances and data independency were evaluated before all the analyses. Results were expressed as means ± S.D. using STATISTICA software (version 8.0).

Results

Phenolic and flavonoid contents

Total phenolics was estimated as 10.5±0.018 mg GAE/g leaf dry weight using the standard curve of gallic acid (r^2=0.9665). Using the standard curve generated by quercetin (r^2=0.995), the total flavonoid contents was measured as 2.44±1.27 mg QE/g leaf dry weight.

Cytotoxic effects of the A. eriophora extract

Fibroblast cells, at passage16, were subjected to MTT assay after treatments with the extracts, at different concentrations (1-512 µg/mL), to test their possible cytotoxicity. While the extracts showed no cytotoxic effects on the cells at lower concentrations up to 2 µg/mL, they proved to be toxic at higher concentrations and the toxicity was dose dependent (Fig. 1). After 24, 48 and 72 h treatment, the half maximal inhibitory concentrations (IC50) values were determined as 180, 50 and 20 µg/mL, respectively.

Proliferation inducing effect of A. eriophora extract

Human fibroblasts in culture showed higher growth rate after treatment with methanolic extracts of *A. eriophora* at low concentrations of 0.1 µg/mL to

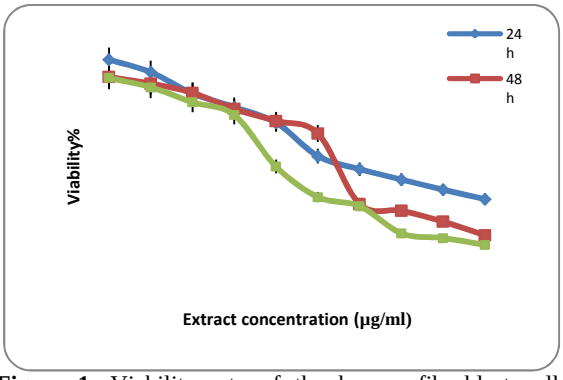

Figure 1. Viability rate of the human fibroblast cells cultured with different concentrations of *A. eriophora* leaf extract, examined by MTT method in different time courses (24, 48 and 72 h). Bars represent the mean±S.D. of 3 wells.

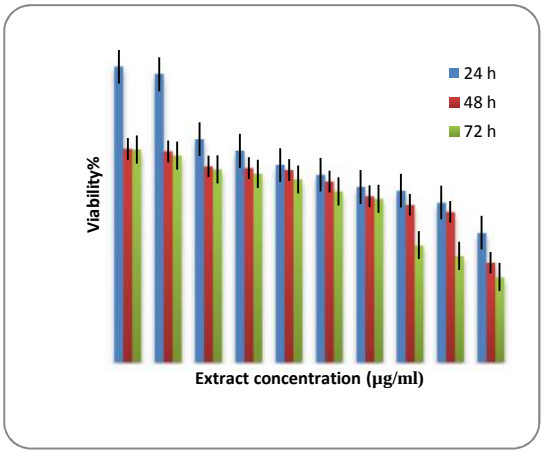

Figure 2. The effect of different concentrations of *A. eriophora* leaf extract on the proliferation of human fibroblasts. The cells were seeded at 8000 cells/well in 96-well plates and assayed by MTT. Bars represent the mean±S.D.of 3 wells.

1.6µg/mL (Fig. 2). Growth stimulatory effects of the extracts decreased by the time and concentration, so that maximum effect (158.7% viability) was observed in the minimal applied concentration (0.1 µg/mL), and 24 h after treatment.

The data from the MTT assays were also verified by direct cell counting analysis of the cells, treated in the same conditions and their correlation were recorded. The results were in line with the data from the MTT assay, with a correlation coefficient of 0.92, confirming the cell proliferative property of the extracts (Fig. 3).

Migration of human fibroblast into in vitro wounds

Upon creation the scratches, representing the wound models, in the cultured fibroblast cells, their initial width were estimated between 700 to 800 µm (Fig. 4).

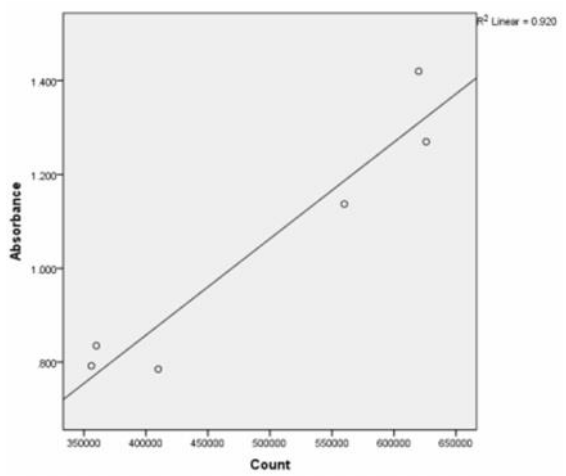

Figure 3. Correlation between the cell number (hemocytometer counts) and data from MTT assay of fibroblasts treated with 0.1 µg/mL of *A. eriophora* leaf extract, 24 h after treatment. These data supports each other (r^2=0.92) at the applied concentration of the extract. Bars represent the mean±S.D. of 3 dishes.

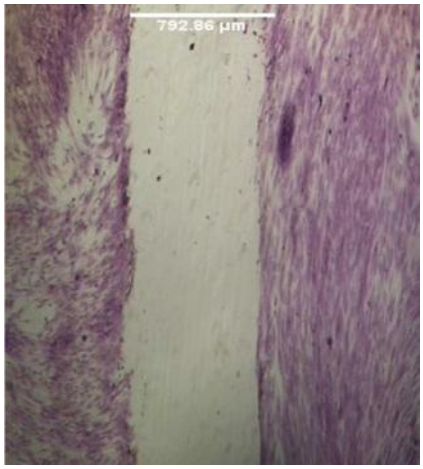

Figure 4. A scratched zone made in cellular monolayers of fibroblasts in culture, representing wound model *in vitro*. Micrograph was made by phase contrast microscopy.

In the DMEM 1% FBS medium, used for this assay, the fibroblast migrated into the denuded area as individual cells, not as a sheet. The migration rate of the cells in the treated groups depended very much on the applied concentrations of the extract, while the scratch in the control groups (DMEM with 1% FBS and without the extract) became static or regressed slightly, after 48 hours. Intermediate concentrations of the extract (from 1 to 40 µg/mL) generally stimulated the migration of fibroblasts into denuded area. *A. eriophora* extract at 1 µg/mL resulted in faster and more complete closure of the scratch. Percentage of closure (calculated

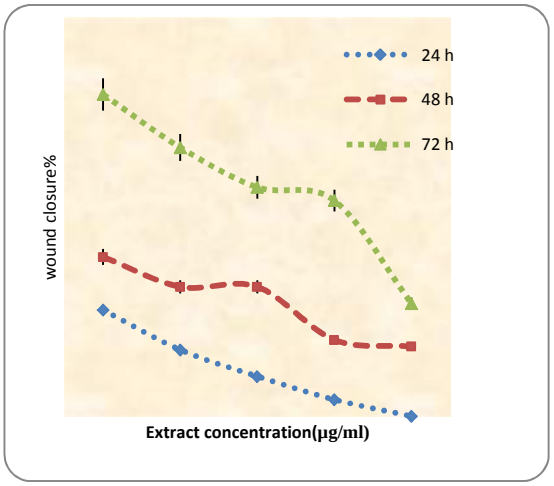

Figure 5. The effect of *A. eriophora* leaf extract on the migration of human fibroblasts into the scratched zone of the monolayer fibroblasts in culture. Values represent the percentage of wound closure (mean±S.D. of 4 wells), calculated by image J. software.

by Image J. software) was 32% by 24 hours post treatment. After 48 h treatment with 1 µg/mL of the extract, the scratch closure was recorded as 48% (Fig. 5). Interestingly, 72 h following the same treatment, the scratch was almost completely closed, forming a scar shape repair in the middle of the denuded area (Fig. 6 e). However, no migration was observed in the control groups, and even the edges of the scratches were deformed (Fig. 6 d). The results indicated that increasing the extract concentration was negatively correlated with the migration rate of the cells (Fig. 5). 24 h after treatment with the maximum concentration (40 µg/mL) of the extract, no obvious migration of the fibroblast was observed (fig. 6 c) However in the same treatment, the scratch closure happened after 48 and 72 h by rates of 20%, and 34%, respectively (Fig. 6 f).

Applying doses higher than 40 µg/mL of the extract, which had no effect on proliferation (Fig. 2), inhibited the movement of fibroblasts, restricting the closure of the scratch compared with controls (data not shown). On the other hand, concentrations lower than 1 µg/mL, in contrast to stimulatory effects on fibroblast proliferation, did not influence migration of fibroblasts into the scratch (data not shown).

Discussion

In severely burned patients, early closure of wounds is one of the vital factors in decreasing the post-burn complications and reducing the mortality rate [15]. Wound healing involves several overlapping phases of inflammation, formation of granulation tissue, re-

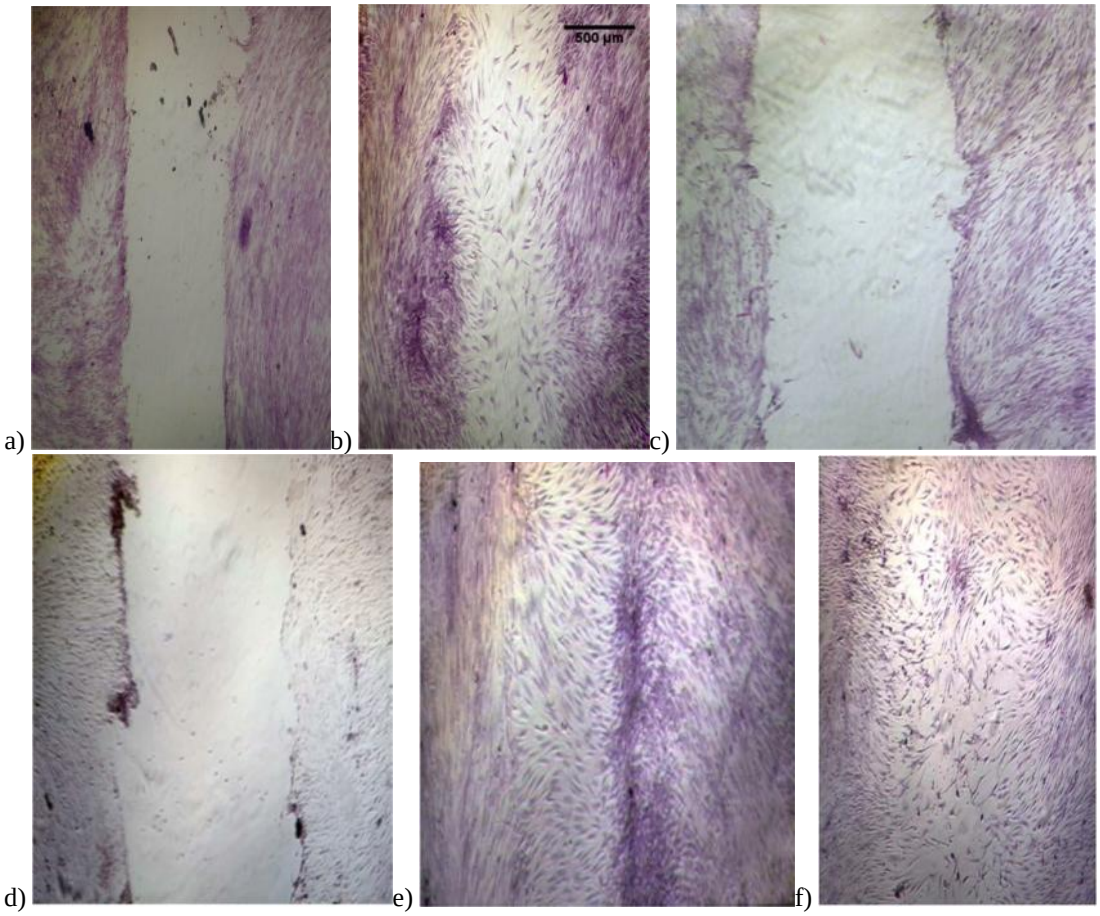

Figure 6. Photomicrograph for *in vitro* wound assay. Fibroblast migration rates were assessed at a time period of 24 h after wound induction in the groups of DMSO control (a), and treatments with *A. eriophora* leaf extract at the concentrations of 1 µg/mL (b) and 40 µg/mL (c), and also 72 h after wounding in the groups of DMSO control (d), 1 µg/mL (e), and 40 µg/mL (f) of the extract.

epithelialization, scar formation, and remodeling [8].

Ethnopharmalogical researches indicated wound healing potential of some *Achillea* species extracts. For instance, the wound healing activity of flower extract of *A. kellalensis* [5], leaves extract of *A. millefolium* [4] and hydroalcoholic extracts prepared from aerial parts of *A. biebersteinii* [16], in an *in vivo* rat model were reported.

In vitro tests are now widely employed in ethnopharmacological studies because of ethical reasons. Moreover, for many disease conditions, cultured cells permit the utilize a variety of *in vitro* tests as the biochemical and biological mechanisms underlying disease and healing processes are understood They also authorize investigation on the influence of pharmacological agents which can modulate this process.

Antioxidant and antimicrobial efficiency of pharmacological agents are known as two biochemical mechanisms underlying wound healing process [17]. Since the damaged skin is highly susceptible to microbial infectivity, antimicrobial factors can accelerate the wound healing process [18]. In the case of oxidants, they are important damaging agents in all stage of tissue repair. For example, H_2O_2 was reported to cause fatal injury to fibroblasts, to block cell signaling by inhibition of epidermal growth factor receptor internalization and to inhibit keratinocyte migration [19, 20]. Free radicals also increase the permeability to pathogens. Overall, antioxidants and every factor that strengthen the antioxidant system, play a significant role in facilitating the wound healing process [21].

Based on recognized antimicrobial [22] and antioxidant [23, 24] properties of *A. eriophora* extract, its wound healing potential can be forecasted. The present study was aimed to investigate the potential activity of this extract in stimulating the proliferation

and migration of fibroblast cells as two another mechanisms for the wound healing efficiency of the extract. Fibroblasts play very important roles in the initial phase of wound healing. Fibroblast cells migrate into the wound site 24 h after injury. During this phase of healing (4 to 21 days), they are activated and undergo a burst of proliferative and synthetic activity, producing high amount of fibronectin, followed by synthesis of other protein components of the extracellular matrix, such as collagen, elastin, and glycosaminoglycans. Fibroblasts also contribute to contraction of the wounds [8].

In the proliferation experiments, after seeding cells in fibroblast growth medium (15% FBS) to ensure recovery from subculture or thawing, the growth media was replaced by medium without supplementation. In these conditions *A. eriohora* extract, at concentrations from 0.1 to 0.8 µg/mL, significantly stimulated human fibroblast growth in monolayer cell cultures, compared to that in control (basal medium).

Chemical composition of *A. eriophora* extract is unknown, but according to the accomplished studies on the *Achillea* genus compounds, flavonoids such as apigenin, rutin, luteolin [25], quercetin, and compherol [26, 27]; and terpenoids such as achillin and piperitone [16] are major fractions of the *Achilea* genus extracts.

Some studies have reported antiproliferative effects for high concentrations of flavonoids (apigenin, luteolin, fisetin and hydroxyflavons) on tumor [28, 29] and normal [30] cells. However there is a report claiming that low concentrations of isolated flavonoids from *Ginkgo biloba* (ginkgetin, rutin, campherol and quercetin) are responsible for the proliferative activity of this extract on the human skin fibroblasts, and that this effect was directly related to the increased extracellular matrix proteins when the cells receive flavonoids [31]. Furthermore, Dinda et al. (2016) reported wound healing activity of *Calendula officinalis* extract by stimulating *in vitro* and *in vivo* proliferation of dermal fibroblasts, highly attributed to the flavonoid contents of the extract [32]. A more delicate evaluation suggested that the impact of flavonoid compounds on the efficiency of fibroblast proliferation depends on their structure, in addition to the concentration. As is reported, short-time pretreatment with proanthocyanidin and catechin could accelerate proliferative response of mouse fibroblasts, but epicatechin gallate, epigallocatechin, and epigallocatechin gallate failed [33].

Considering prior reports, the results of present study tend to suggest that the proliferative activity of low concentrations of *A. eriophora* extract on the human fibroblasts may be related, somehow, to the flavonoid contents of the extract.

Fibroblast migration is a major event during the process of wound healing which may sometimes be arrested, even when there is development of good granulation tissue. Migration is influenced by the nature of surrounding extracellular matrix and protein composition of the wound environment. In the 'scratch' model, used in this study, the medium concentrations of *A. eriophora* extract (1 to 40 µg/mL) normally led to migration of the cultured fibroblasts and efficient closure of the scratched zones. A clear negative correlation between the increasing concentrations of the extracts and the degree of cell migration can be observed.

Previous studies of flavonoids on the various cell lines have summarized on their inhibitory effects on cell migration [34, 35]. However, some new researches have been focused on flavonoids, as inducing factors on normal cell migration [36]. Namely, a phytochemical study indicated stimulative effect of 100 flavonoid types on human keratinocyte migration, also presented a relationship between this activity of flavonoids and their biochemical structures [37]. Furthermore, a recent evaluation on the soybean flavonoids demonstrated promoting effect of glycitin (4'-hydroxy-6-methoxyisoflavone-7-d-glucoside) on the human dermal fibroblast cell proliferation and migration, via increasing expression of transforming growth factor-beta [3]. On the other hand, in a related study pure antioxidants such as phenolics have been introduced as promoting factors for proliferation and migration of fibroblast cells, in lower and higher concentrations, respectively [40]. Accordingly, it is proposed here that the migratory potential of the medium concentrations of *A. eriophora* extract on the human fibroblasts may be related to the nature of flavonoid compounds of the extract.

As already demonstrated, regulation of fibroblast proliferation and migration are governed by independent mechanisms [41, 42]. This difference was also evident in the current study, by a biological model of *in vitro* wound assay. While 0.1 µg/mL of the extract markedly stimulated human fibroblast proliferation, it did not show significant effect on closure of the scratches, and that 1 µg/mL concentration which had no significant effect on the cell proliferation, was the most effective dose on stimulating of migration in the fibroblasts.

In conclusion, to the best of our knowledge, we established an *in vitro* model for assessment the wound healing activity of the methanolic extract of *A. eriophora* for the first time. According to the obtained results, 0.1 and 1 µg/mL of the examined extracts were the most effective doses for the stimulation of

proliferation and migration of the human fibroblasts, respectively. From these data plus the fact that these doses did not show any cytotoxic effects on the human fibroblast cells, the crude methanolic leaf extract of *A. eriophora* is highly recommended as a potential source for improving the wound healing activity in skin. However, the exact mechanism by which this extract exerts the stimulations and the responsible components for such actions, remain to be elucidated. Also further biochemical researches are needed to recognize and isolate the active compound(s) responsible for these pharmacological activities.

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