Journal of Stem Cells & Regenerative Medicine
ISSN 0973 - 7154

ORIGINAL ARTICLE

JSRM Code: 018020300006

Aqueous extract of *Achillea millefolium* significantly affects mesenchymal stem cell renewal and differentiation in a dose dependent manner

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Abstract

Introduction: *Achillea millefolium* is an Iranian herbal medicine with various effects on the human cells. The aim of this study was to investigate the effects of the aqueous extract of *Achillea millefolium* (AEAM) on the proliferation and differentiation of mesenchymal stem cells (MSCs).

Methods: In this study, bone marrow-MSCs (BM-MSCs) were obtained from Wister rat bone morrow and then cultured in Dulbecco's modified Eagle's medium /Nutrient Ham's Mixture F-12 (DMEM/F12) media. Then, the isolated MSCs were cultured in either osteocyte or adipocyte differentiation media containing 0.2 or 2 mg/mL AEAM and assessed using specific staining method.

Results: The isolated BM-MSCs exhibited fibroblast-like morphology and were positive for CD73, and CD90, while negative for CD34 and CD45. AEAM significantly increased self-renewal of BM-MSCs at low dose (0.2 mg/ml, P= 0.001) and increased the pool stem cells in both osteocyte and adipocyte differentiation media.

Conclusion: AEAM at low doses may be used in cases where there is a need for large number of stem cells, via increased numbers of MSCs, and help tissue repair and immunomodulation.

Keywords: *Achillea millefolium*; Mesenchymal stem cell; Differentiation; Proliferation; Inflammation

Introduction

Nowadays, herbal medicines are widely used in developing countries in treatment of several human diseases[1]. Although an old idea expresses that the chemical medicines are associated with various adverse effects, there are limited scientific reports regarding the side effects of the herbal medicines on eukaryotic cell systems[2]. *Achillea millefolium* is widely used as a well-known herbal medicine in Iranian traditional medicine[3, 4]. Accordingly, it is used to relieve the symptoms of stomachache, grippe, sinusitis, insomnia, muscle spasms and gastrointestinal disorders[3, 4]. However, it has been reported that excessive or long-term use of this herbal medicine may be associated with vertigo and headache[5]. Nevertheless, further research is needed to determine the benefits and side effects of *Achillea millefolium*.

Mesenchymal stem cells (MSCs) derived from different tissues such as adipose, bone morrow, dental pulp and placenta have been reported as potential cells with the ability to differentiation into the adipocytes, osteocytes and chondrocytes[6]. Additionally, MSCs have immune-modulatory potential and play key roles in several inflammation-related diseases[7]. Therefore, environmental factors affecting these cells may interfere with the self-renewal and differentiation potential of MSCs.

Because of the need to clarify the effects of Achillea millefolium on MSCs, this project was designed to explore the effects of Aqueous extract of Achillea millefolium (AEAM) on the self-renewal and differentiation of rat BM-MSCs.

Materials and Methods

In this project, MSCs were obtained from a Wistar rat bone morrow and then cultured in Dulbecco's modified Eagle's medium /Nutrient Ham's Mixture F-12 (DMEM/F12) media. Then the BM-MSCs were cultured in both osteocyte and adipocyte differentiation media on either 0.2 or 2 mg/mL AEAM. The differentiations were assessed using the specific stained method. The protocol is presented in detail as follows

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doi:10.46582/jsrm.1802006

Received 15 Nov 2021; Accepted 15 Sep 2022; Published online [epub ahead of regular issue]: 21 Nov 2022

JSRM/Vol.18 No.2, 2022; P29

2.1 Preparation of aqueous extract of Achillea millefolium

The fresh plant was purchased with specimen no. 9757 from Isfahan Botany Herbarium Company, Isfahan, Iran. After that, the flowering branches were removed, washed, dried and then grounded. The ground powder (2 gr) was percolated with distilled water (200 mL) for 24 h and then filtered by using Whatman filter paper (8–10 µm). The filtrated solution was evaporated yielding dried extract to prepare AEAM. To prepare the work solution, 20 mg of the AEAM was dissolved in 1 mL of distilled water and then 100 and 10 µL per 1 ml of the media were added to both the osteocyte and the adipocyte differentiation media to make 2 and 0.2 mg/mL AEAM, respectively.

2.2 Isolation and culture conditions of mesenchymal stem cells

In this project, a six- to eight-week-old male Wistar rat was sacrificed by cervical dislocation in the standard and ethical conditions.

According to our previous study[6], the femurs and tibiae were aseptically dissected and used for MSCs isolation by flushing with Phosphate Buffer Saline (PBS), supplemented with 10% antibiotic-antimycotic (Sigma-Aldrich). Then, the pooled suspension of bone marrow-derived cells was prepared in Dulbecco's modified Eagle's medium/Nutrient Ham's Mixture F-12 (DMEM/F12) media containing 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and filtered through a 70 µm nylon cell strainer. The cells were then seeded in a 25 cm^2 flask at a density of 1-2 × 10^6 cells/cm2 and kept in a humidified 5% CO2 incubator at 37 °C. After 24 h, non-adhesive cells were removed by washing with PBS and 5 ml fresh culture medium was added. Thereafter, colony-forming attached cells were cultured and the medium was replaced every three days. Upon reaching 70–80% confluence, BM-MSCs were passaged and the 3–5 generation cells were used for the experiment.

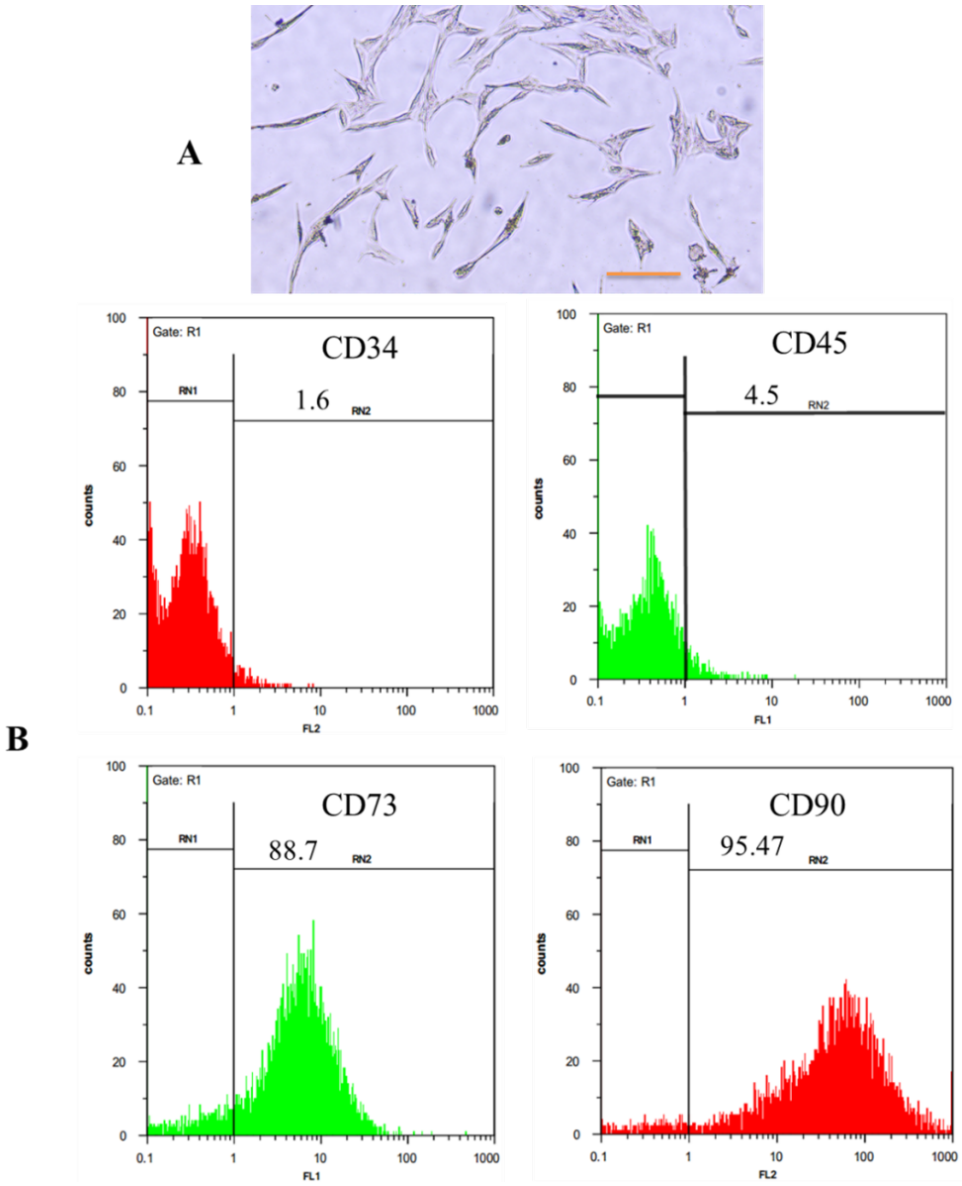


Figure 1: Characterization of BM-MSCs. (A) fibroblast-like appearance of the cultured mesenchymal stem cells (×100, Scale bar = 50 µm). (B) Flow cytometry of BM-MSC specific CD markers. Representative histograms showed that BM-MSCs were positive for CD73 and CD90, and negative for CD34 and CD45.

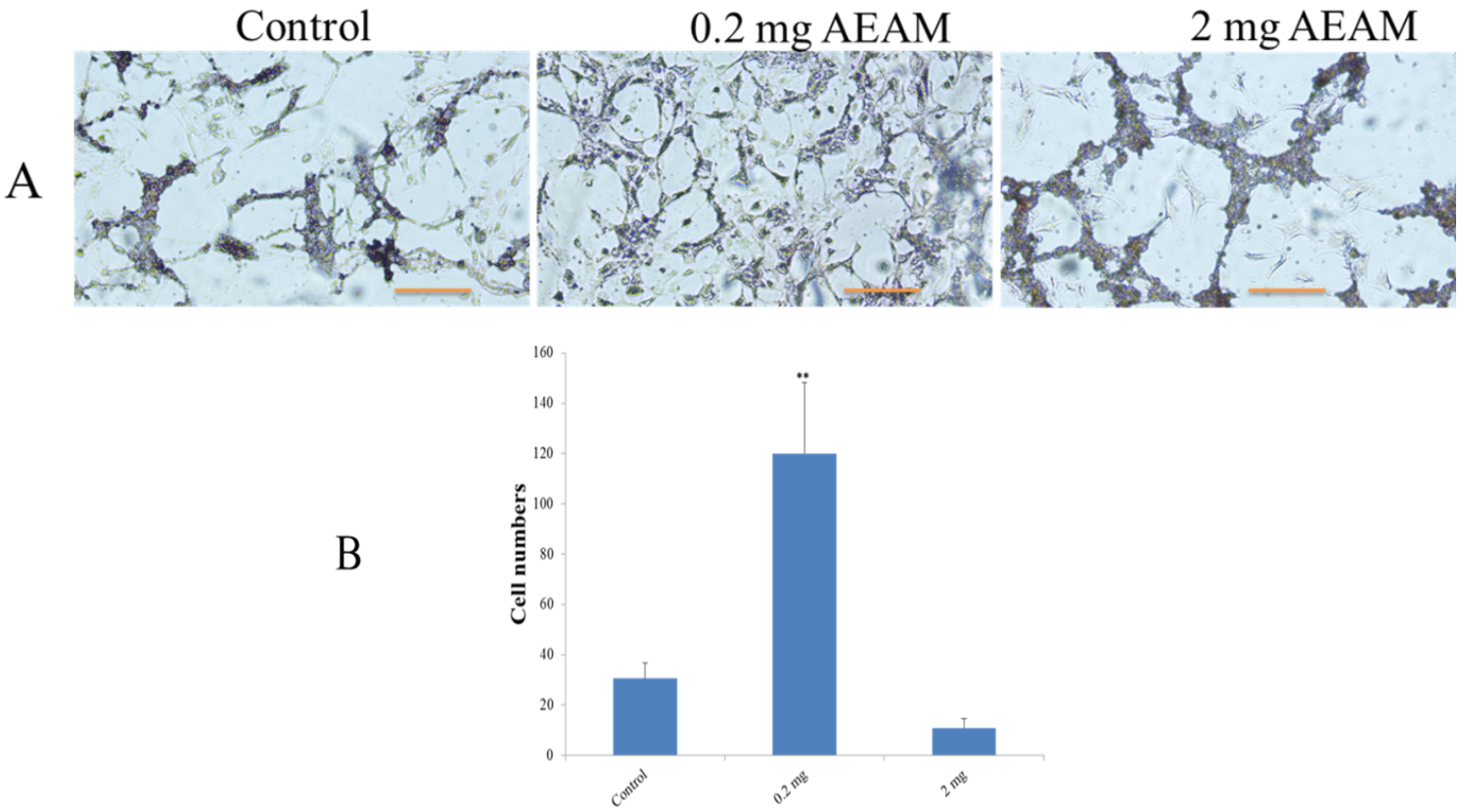
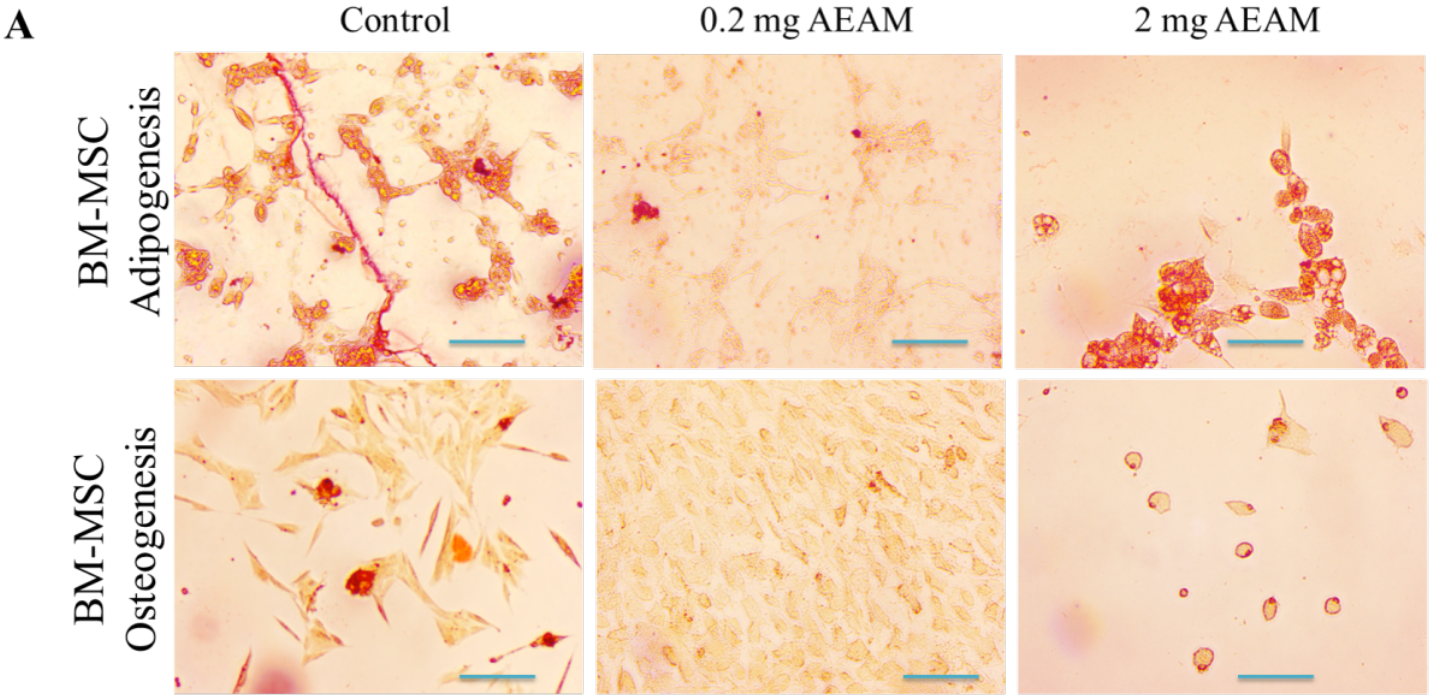


Figure 2: (**A**) Inverted microscope images of BM-MSCs after 21 days of differentiation induction in experimental groups (×100, Scale bar = 50 µm). (**B**) Cell numbers of BM-MSCs after 21 days of differentiation induction. Five visual fields were selected randomly to calculate the number of MSCs, and the results showed that the number of cells in 0.2 mg/mL AEAM was higher than control and 2 mg/mL AEAM groups (P = 0.001). Error bars indicate the SD on the mean normalized data



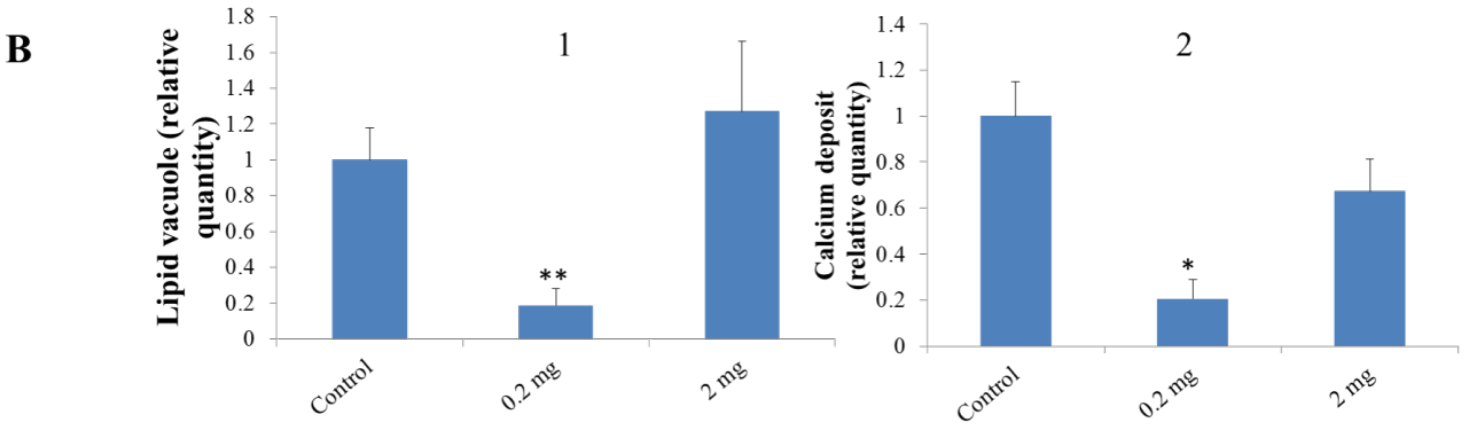


Figure 3: The effects of AEAM on the adipogenic and osteogenic differentiation of mesenchymal stem cells. After 21 days of adipogenic and osteogenic differentiation, lipid droplet and matrix mineralization were stained using Oil Red and Alizarin Red S Staining, respectively. Figure 3A illustrates that AEAM in low concentration (0.2 mg/ml) significantly decreased MSC adipogenic and osteogenic differentiation, while AEAM in high concentration (2 mg/ml) significantly increased MSC adipogenic and osteogenic differentiation (×100, Scale bar = 50 µm). (B) Positive Oil Red (B1) and Alizarin red S (B2) staining were quantified by Image J analysis software. Error bars indicate the SD on the mean normalized data.

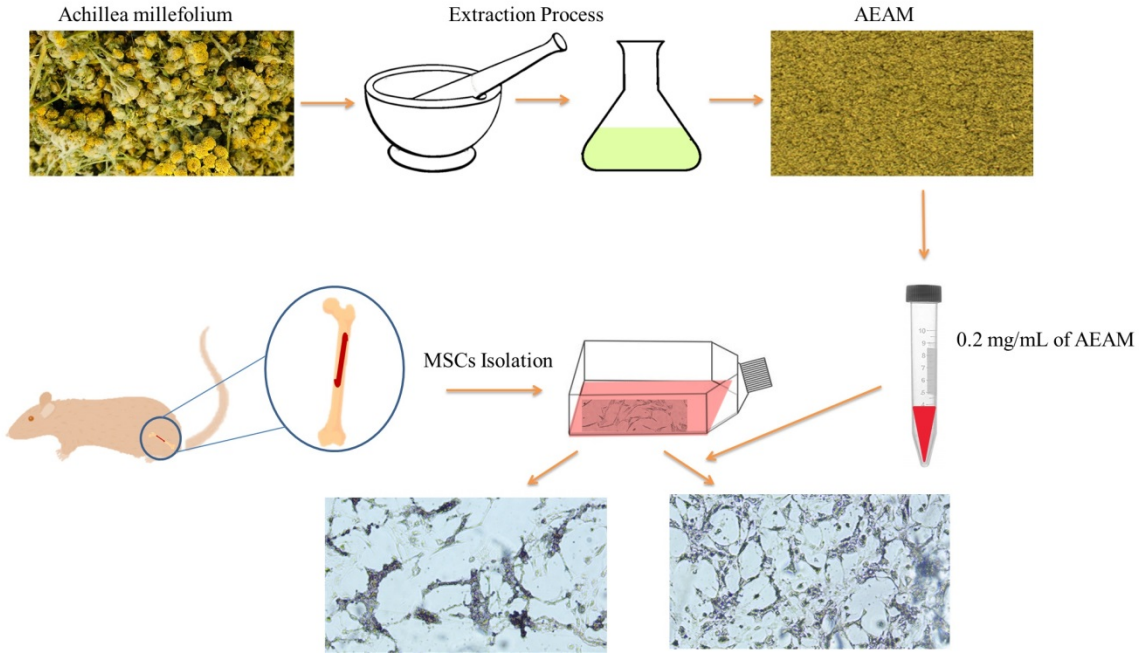


Figure 4: Schematic illustration of study design. Preparation of aqueous extract of Achillea millefolium (AEAM) and its effects on rat bone marrow-derived mesenchymal stem cell renewal and differentiation.

2.3 Characterization of BM-MSCs

2.3.1 *Assessment of MSC surface markers*

To confirm the MSCs, cell surface markers were examined using flow cytometry technique. The cells at the first passage were used for staining with fluorescence-labeled anti-CD34, CD45, CD73, CD90 (eBioScience, San Diego, CA) antibodies. Accordingly, the cells were washed twice with PBS and incubated with the antibodies for 30 min and then analyzed using a Beckman Colter flow cytometer and FACScan program (US). Characterization data of BM-MSCs are presented in Figure 1.

2.3.2 *Multipotency assessment of MSCs*

For evaluating the BM-MSCs multipotency capacity, the cells were seeded in 12- well plates and incubated in complete culture osteogenic and adipogenic differentiation media (Stem Cell Technology Company, Bon Yakhteh-Tehran, Iran). The culture media were replaced with fresh differentiation media every 3 days. After 3 weeks, the cells were washed with PBS and fixed in 4% PFA (paraformaldehyde, Sigma). The adipogenic differentiation of BM-MSCs was demonstrated by Oil Red O (Sigma Aldrich) staining solution (1%) for detection of triglyceride or lipid vacuoles. The osteogenic differentiation of BM-MSCs was checked by Alizarin Red S staining solution (4%) for detection of calcium deposition.

2.4 AEAM and MSCs differentiation potential

Multipotency assessment of the BM-MSCs and the effects of AEAM on the differentiation potential of the BM-MSCs were determined by culturing in osteogenic and adipogenic media (Stem Cell Technology Company, Bon Yakhteh-Tehran, Iran). The BM-MSCs were cultured in three conditions, without AEAM, with 0.2 mg/ml and with 2 mg/mL of AEAM. In these conditions, the cells were incubated for 21 days, and then fixed using 4% paraformaldehyde. The fixed cells were stained using Oil Red O (Sigma Aldrich) and Alizarin Red S (Sigma Aldrich) to determine the adipogenic and osteogenic differentiation, respectively.

2.5 **Statistical analysis**

Statistical analysis was performed using SPSS Statistics V22.0 (IBM, USA). Data were presented as the mean ± standard deviation (SD). One-way ANOVA was utilized to determine the statistical significance. A p-value ≤ 0.05 was considered statistically significant.

Results

BM-MSCs exhibited typical fibroblastic morphology and could attach to plastic (Figure 1A). Flow-cytometry analysis showed MSC-specific markers, CD73 and CD90, were strongly expressed in the BM-MSCs while the expression levels of CD34 (hematopoietic progenitors) and CD45 (lymphocyte common antigen) were not detectable (Figure 1B).

The results demonstrated that AEAM in 0.2 mg/ml significantly induced self-renewal and decreased differentiation of the BM-MSCs in either osteogenic or adipogenic differentiation media. Accordingly, AEAM in 0.2 mg/ml significantly increased BM-MSCs proliferation, when compared to control group ($P< 0.01$) (Figure 2). 0.2 mg/ml AEAM significantly decreased adipogenic ($P< 0.01$) and osteogenic ($P< 0.05$) BM-MSCs differentiation (Figure 3).

While BM-MSCs proliferation was not significantly different between AEAM in 2 mg/mL and control group (Figure 2). AEAM in 2 mg/ml increased and decreased differentiation of the BM-MSCs in adipogenic and osteogenic differentiation media respectively, but comparisons between the group and the control group were not statistically different (Figure 3).

Discussion

In regenerative medicine, MSC is one of the most frequently used cell types with therapeutic potential in treatment of different pathologies, including brain damage[7], immune-mediated disorders[8], respiratory diseases[9], neurological disorders[10], cardiac ischemia[11], diabetes[12], bone and cartilage diseases[13], digestive system[6], cancer[14], COVID19[15].

In this study, the results demonstrated that AEAM significantly affects proliferation of BM-MSCs independent of differentiation media conditions. Accordingly, low AEAM doses led to induce BM-MSCs renewal. In addition, using low AEAM doses was associated with decreased rate of differentiation into osteocyte and adipocyte. Our results are consistent with the fact that there is an inverse association between cell proliferation and differentiation, and the high proliferative capacity usually coincides with low differentiation capacity. As MSCs are one of the main cell sources of the body to repair tissues and for regulation of immune responses, hence it may be hypothesized that using AEAM at low doses can be suggested to achieve specific aims. For example, in cases of tissue repair aims, such as bone fracture, using low doses of AEAM may be associated with elevating the MSC pool and then, accelerated recovery.

In contrast to the high doses, the low doses were associated with proliferation of BM-MSCs in both osteogenic and adipogenic differentiation media. Therefore, it may be used in the cases that a number of MSCs are needed to control the diseases, including the pro-inflammatory related disorders. However, the proposed use of the AEAM at low and high doses is theoretical and needs to be examined in the *in vivo* conditions. To the best of our knowledge, this is the first investigation regarding the effects of AEAM on the MSCs proliferation and differentiation. The study by Ghobadian et al. had the same results on the human skin fibroblast cells (HSF-PI-16). They reported that AEAM at low doses led to increase and at high doses was associated with decreased proliferation of HSF-PI-16[16]. Therefore, it may be hypothesized that the effects of AEAM on the differentiated cells is similar as the multipotent cells, such as MSCs.

The effects of AEAM on cancer cells seem to follow a different pattern. A study on the cancer cell lines has found that AEAM significantly decreases cell proliferation in HeLa cervical cancer cell line[17]. Therefore, it seems that low doses of AEAM can be associated with increased and decreased proliferation of BM-MSCs and cancer cells, respectively. Therefore, it seems that low-dose AEAM can be considered as a safe herbal medicine.

Some bioactive compounds in herbal medicines have been shown to increase the rate of cell proliferation and differentiation through complex signal pathways like BMP2, Runx2, and Wnt[18]. Research has shown that several extracts and natural compounds from herbal medicines regulate MSC genes expression, which may be responsible for the MSCs proliferation and multilineage differentiation[18]. Stem cell differentiation and proliferation are regulated by Wnt signaling pathway[19].

Herbal medicine extracts contain a variety of bioactive compounds like polyphenols, flavonoids, and many other compounds and chemical substances[18]. MSCs in combination with herbal medicine extracts confer a potential hope in stem cell and regenerative therapy[18]. Achillea millefolium promotes the proliferation of BM-MSCs similar to bioactive compounds such as flavonoids, soy peptides, polysaccharides, polyketides and curcumin[18].

Herbal medicines have the potential to improve MSCs proliferation and differentiation and can develop a cost-effective technology for cellular therapy[18].

Based on the immunoregulatory capacity of the MSCs, they can be used as an effective cell-based therapeutic strategy for inflammatory and autoimmune conditions. There are several investigations regarding the anti-inflammatory effects of AEAM in the *in vivo* condition. For instance, our previous investigation revealed that AEAM led to decrease of disease severity and pro-inflammatory responses in EAE-induced mice[20]. Pirzad Jahromi and colleagues reported that AEAM at 7 mg/kg significantly restored memory deficit and decreased pro-inflammatory related anxiety that were caused by stroke in an animal model[21]. Another study on the atopic dermatitis-like skin lesions in NC/Nga mice revealed that AEAM significantly decreased the levels of pro-inflammatory cytokines[22]. Based on the effects of low doses of AEAM on the BM-MSCs, it may be hypothesized that AEAM inhibits pro-inflammatory responses via increased renewal of the MSCs. However, it needs to be explored in the *in vivo* conditions.

Moreover, AEAM can be useful in the case of tissue damages, because of its potential to maintain MSCs pools. To the best of our knowledge, there are no investigations regarding the effects of *Achillea millefolium* extract on the osteocyte and adipocyte differentiation of MSCs. Additionally, since AEAM at low doses is associated with an increased number of MSCs, it may lead to acceleration of tissue repair. Thus, the authors of the current study propose to perform some projects regarding the effects of *Achillea millefolium* extract at low doses to explore the effects of this plant extract on the tissue repair.

Conclusion

In conclusion, AEAM at low doses can increase the BM-MSCs proliferation and has the potential to maintain MSCs pools in body. In addition, the use of AEAM at low doses is suggested to obtain a sufficient number of MSCs for stem cell therapy. The results of this study and further research can provide important experimental evidence for the AEAM administration as an adjuvant treatment to increase the effectiveness of MSC-based therapeutic strategies.

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Funding agency

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Conflicts of Interests

None

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