International Journal of Cellular & Molecular Biotechnology 2014 (2014) 1-10

ISPACS
International Scientific Publications and Consulting Services

Available online at www.ispacs.com/ijcmb
Volume 2014, Year 2014 Article ID ijcmb-00012, 10 Pages
doi:10.5899/2014/ijcmb-00012
Research Article

Effects of *Crocus sativus L.* extract and vitamin D3 on in vitro osteogenesis of mecenchymal stem cells

Baharara Javad[1,3*], Ramezani Tayebe[2], Nejhad Shahrokhabadi Khadijeh[3], Nazemi Maryam[3]

(1) Research Center for Animal Development Applied Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran
(2) Department of Animal Biology, Kharazmi University, Tehran, Iran
(3) Department of Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran

Abstract

Studies on stem cells have created new possibilities for osseous defects therapies. This expremental study focused on the synergic effects of saffron extract and vitamin D3 on osteogenesis differentiation in bone marrow mesenchyme stem cells (BMSCs) of wistar rat. BMSCs were isolated from rat's femur and treated with different doses of saffron extract, vitamin D3 and combination saffron extract and vitamin D3. Extract cytotoxicity was measured using MTT assay. The alkaline phosphatase (ALP) enzyme activity, Alizarin red staining and RT-PCR were performed to evaluate the differentiation progress. MTT assay indicated saffron extract, vitamin D3 and a combination used of those factor decreases cell viability. ALP activity showed that saffron extract, Vitamin D3 and application of two factors together increase ALP activity. Alizarin red staining showed vitamin D3, saffron and synergic used of them affects cell mineralization. RT-PCR result demonstrates in 14 days the group that treated with vitamin D3 and saffron Together express osteocalcin gene.

Keywords: Osteogenesis, saffron, rat, vitamin D3, osteocalsin

1 Introduction

The treatment of serious osseous defects remains a great challenge in orthopedic surgery [1]. however Studies on pluripotent stem cells for this proposes have created cell-based therapies for osseous defects Because the use of human embryonic stem cells is ethically controversial, focus has shifted to the use of adult stem cells, especially BMSCs [2]. BMSCs are a population of self-renewing pluripotent cells that have a potential in cellular therapies for tissue generation [3]. BMSCs can differentiate along several lineages, such as the osteogenic lineage, in response to stimulation by environmental agents [4].To

* Corresponding Author. Email address: baharara@yahoo.com, Tel: +985118437092, Fax: +985118437092

increase the differentiation of stem cells into bone cells, growth factor including platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGF-β) showed promisingly effects [5-7]. However, the high cost and rapid degradation of such expensive growth factor limited their used [6]. Therefore, there is an essential need to expand some alternative osteogenic inducer premier capacity and lower costs than growth factor [4]. In recent years increased regard to use of natural products especially medical plant in medicine. The cellular components of bone contain of osteogenic precursor cells, osteoblasts, osteocytes and osteoclasts of bone marrow [8]. Osteoblasts are metabolically active, bone forming cells. They secrete osteoid, the unmineralized organic matrix that subsequently undergoes mineralization,giving the bone its strength and rigidity. As their bone forming activity nears completion, some osteoblasts are converted into osteocytes [9]. Osteoblast express alkaline phosphatase, early osteogenic marker, and late markers of differentiation such as osteocalcin and osteopontin [10] Osteocalcin is a bone-specific protein synthesized by osteoblasts that represents a good marker for osteogenic maturation [11]. 1α,25-dihydroxyvitaminD3 (1,25D) increase the osteogenic differentiate in multipotent mesenchymal stem cells. This vitamin acts on osteoblasts via classical vitamin D receptors (VDR) and via a membrane 1,25D-binding [12]. vitamin D3, is a secosteroid hormone that not only regulates bone and calcium/phosphate metabolism but exerts a number of other biological activities [13]. Crocus sativus L. commonly known as saffron, is a perennial stemless herb of the Iridaceae family and widely cultivated in Iran and other countries such as India and Greece [14]. Saffron contains chemical constituents such as crocetin, picrocrocin and safranal which are responsible for color, flavor and aroma respectively. Anthocyanins, flavonoids, vitamins, amino acids, proteins, starch, minerals and other chemical compounds have also been described in saffron [15]. The goal of this study was investigate effects of saffron extract and vitamin D3 on invitro osteoganic differentiation of bone marrow stem cells.

2 Materials and methods

Preparation of plant extract: *Crocus* sativus L. stigmas were collected from Ghaen (Khorasan province, Northeast of Iran). Dried and powdered stigma of Saffron was resolved in Distilled water (Kimia, Iran) and soaked 24 h in times at 4˚C. The extract was filtered and concentrated with a rotary evaporator and dried with frizz drier. The extract then dissolved in deionized water to a final concentration of 100 mg/ml and diluted in culture medium to the working solution before use. The extract was filtered and stored at 4 °C.

Preparation of vitamin D3: Vials of injectable vitamin D3 Was purchased from drug stores and was dissolved in The dimethyl sulfoxide (DMSO)(Merck. Germany) and was diluted in culture medium.

Isolation and Expansion of BMSCs: Bone marrow was harvested by flushing the femurs of 6-8 weeks old rats with DMEM(Sigma, Uk), then this cells were centrifuged, and suspended in DMEM with 15% FBS. Cells were incubated at 37°C in 5% humidified CO2 for 24 h until the medium was replaced to remove non-adherent cells. When cells reached 90% confluence, they were trypsinized (Sigma, uk), with 0.25% trypsin for 5 min at 37°C. After centrifugation, cells were re-suspended and use for other analysis [16].

Immunofluorescence (IF) analysis: For detection the describe isolated cells are stem cell Immunofluorescence (IF) analysis done. After 4 passage the cells were detected as described sun et al [16] Briefly, cells that adhered to the glass slides were washed twice with cold PBS (1x), and fixed in 4% paraformaldehyde (Merck. Germany) for 20 min at 4°C. Following three washes with PBS (5 min each), cells were permeabilized with 0.2% Triton X-100(Merck. Germany) for 30 min, and blocked in 2% BSA (Merck. Germany) for 30 min. Then, the cells were incubated overnight at 4°C with rat's monoclonal antibody CD 44 (1:200) (Abcam, Germany). The cells were then washed with PBS three times (5 min

each) and incubated for 50 min at room temperature with FITC-conjugated goat anti-rat IgG secondary antibody (1:2000) (Sigma, Uk), Subsequently, the cells were stained with diamidinophenylindole (DAPI) (Merck. Germany) for 5 min at room temperature and observed under a fluorescence microscope (labomed, korea).

In vitro cytotoxicity study and cellular: The *in vitro* cytotoxicity was studied using the MTT assay. The cleavage and the conversion of the soluble yellowish MTT(Sigma, Uk), to the insoluble purple formazan by active mitochondrial dehydrogenase of living cells has been used to develop an assay system alternative to other assays for measurement of cell proliferation. Harvested cells were seeded into a 24-well plate (4×10^5 cell/ml) with different amounts OF saffron extract(600.700 and 800 µg/ml), vitamin D3(10^{-7},5×10^{-7} and 10^{-6} M) and These two substances together (800 µg/ml plant extract pulse 10^{-7},5×10^{-7} and 10^{-6} M D3vitamin) for 24 h. in the end of the incubations, 50 µL of MTT solution (5 mg/mL in PBS) was added to each well and left for 4h in dark conditions. Subsequence, the insoluble formazan produced was dissolved in a solution containing 1 ml of DMSO and left for 20 min at room temperature. Finally, the solution optical density (OD) was read with multi well scanning spectrophotometer (Biotek Epoch, US) at a wavelength of 570 nm. The cell viability was calculated using the following equation: Cell viability (%) = $(A_{treated}/A_{control}) \times 100$ where $A_{treated}$ and $A_{control}$ are the absorbance of the treated and untreated cells, respectively.

Osteogenic differentiation and treatment with extract of saffron stigma: BMSCs were seeded on to 24-well plates (5×10^4). When over 80% confluency was reached, osteogenesis was induced by saffron extract(600,700 and 800 µg/ml), vitamin D3(10^{-7},5×10^{-7} and 10^{-6} M) and These two substances together (800 µg/ml plant extract pulse 5×10^{-7} M vitamin D3). The medium was replaced every 3 days. On days 10,15 and 21, cells were collected for measuring ALP activity, reverse transcribed polymerase chain reaction analysis (PCR and alizarin red staining respectively). Triplicate tests were conducted in each experiment.

Alizarin red staining: Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. After washing once with Distilled water, 1 ml alizarin red solution (Merck, Germany) was added to each well in a 24-well plate. The staining solution was removed after 10 min. later each well was washed with Distilled water for four to five times. Fainally observed with invert microscopy (Bio med, Korea).

Measurement of alkaline phosphates (ALP) activity: BMSCs were cultured in 6 well plate dishes for 24 h as described above and then treated with Saffron extract (600,700 or 800 µg/ml), vitamin D3 (10^{-7},5×10^{-7} and 10^{-6} M) and These two substances together (800 µg/ml plant extract pulse D3vitamin (10^{-7},5×10^{-7} or 10^{-6} M) for 10 days. To determine the level of alkaline phosphatase activity, total cell protein was extracted using 200 µl NP40 buffer (Sigma, UK); The lysate was then centrifuged at 14,000×g at 4 ºC for 15 min. Supernatant was collected and ALP activity was measured with ALP assay kit (Parsazmun, Iran) using p-nitrophenyl phosphate (p-NPP) as substrate and alkaline phosphatase provided in the kit as standard. The activity of enzyme (IU) was normalized against total protein content on cell lysate.

Total RNA isolation and (RT-PCR):
Osteocalcin mRNA expression were analyzed with RT- PCR, for this propose RNA was extracted from approximately 30×10^5 MSCs, that were treatment with vitamin *D3* (5×10^{-7}M), saffron extract(800 µg/ml) and synergist used of them(10^{-6} M vitamin D3 plus 800 µg/ml) for 2 weeks using RNEasy per the manufacturer's instructions. The mRNA was reverse transcribed to CDNA using Advantage RT-for-PCR per the manufacturer's instructions. cDNA was amplified using a trmocycler (Perkin Elmer Applied Biosystems, Boston, MA) at 94°C for 40seconds, 56°C for 50 seconds, and 72°C for 60 seconds for 35

cycles, after initial denaturation at 94°C for 5 minutes. Primers used for amplification are Forward primer GTGCAGAGTCCAGCAAAGGT Reverse primer CGATAGGCCTCCTGAAAGC (length = 202). The PCR products were analyzed by the electrophoresis of samples in 1.5% agarose gels stained with ethidium bromide. Osteocalcin gene expression was normalized to B-actin expression in each sample, Forward CCC GCC GCC AGC TCA CCA TGG, Reverse AAG GTC TCA AAC ATG ATC TGG GTC.

Statistical analysis: Data are expressed as mean±standard deviation (SD). Statistical significances were analyzed using the ANOVA test. $p<0.05$ was considered significant.

3 Result

Cells Observation: By 4 to 8 hours after passage, the cells were adherent. Twenty-four hours later, the adherent cells showed a fusi form shape. Three to five days later, the cells increased and showed directionality in their arrangements.

Identification of BMSCs: The cells at the Third passage was examined with Immunocytochemical staining agonist CD44 antigen. Result indicated All cells were highly positive for the surface antigens CD44 (Figure 1).

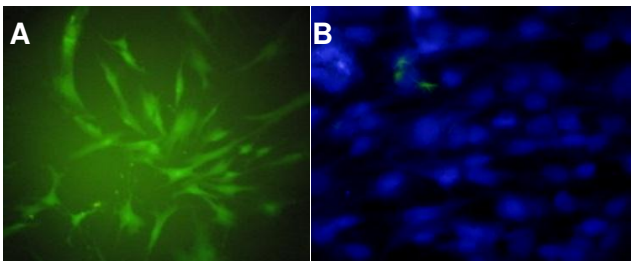

Figure 1A: Immunocytochemical staining BMSCs cells for CD44 antigen, Green fluorescent colour observed in on the cells Due to the presence of CD44 antigen on the surface of these cells. Figure1B: DAPI staining. Cell's Nuclei are observed in fluorescent blue colour.

Cytotoxicity of saffron extract: The BMSC viability values were around 80% when treated with saffron extract (800 µg/ml), vitamin D3 treatment group(10^{-7},5×10^{-7} and 10^{-6})showed decrease in viability in dose depend manner. in groups that treated with plant extract (800 µg/ml)pulse vitamin D3 (10^{-7},5×10^{-7} and 10^{-6} M) Viability in compared with the use of vitamins alone were improved. In overall, the Cytotoxicity assay demonstrated that the viability in all groups (plant extract, vitamin D3 and synergic effect of them) were Greater than the IC50 value (Fig 2).

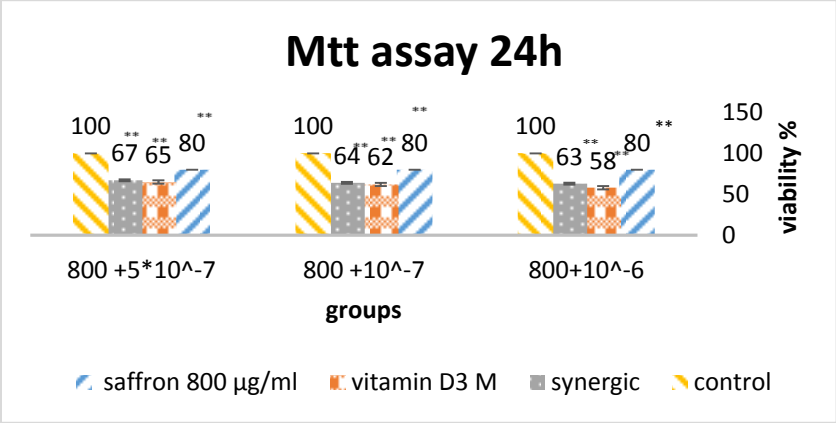

Figure2: Toxicology of different concentrations of plant extract, vitamin D3 and synergic effect of them toward BMSCs (** P < 0.01, ANOVA, ments+S.D)

Alizarin red staining results:

Alizarin red staining (in the groups that are treated with Saffron): The result showed saffron extract can differentiate BMSCs into osteoblasts. The maximum morphological changes and extracellular matrix staining take place in 800 µg/ml doses of plant extract (Fig. 3 B).

Alizarin red staining (in the groups that was treated with vitamin D3): Samples stained with Alizarin red showed that vitamin D3 able to induce differentiation in *BMSCs.* Most difference was observed in the 5×10^{-7} M group compared to the control group (Fig. 3 C).in 10^{-6} M oF vitamin D3 mineralization clearly higher than other group However in this doses Increased cell mortality thus that seems appropriate dose of vitamin D3 alone, to induced osteogenic differentiation is 5×10^{-7} M.

Alizarin red staining (in the groups that was treated with vitamin D3 and saffron extract): Treated BMSCs with saffron extract (800 µg/ml) and different doses of vitamin D3 (10^{-7}, 5×10^{-7} and 10^{-6} M) showed that synergic application of 800 µg/ml of saffron and 10^{-6} M vitamin D3 has maximum effect on osteoblastic distinction of BMSCs compared with the use of each item alone (Fig.3 D).

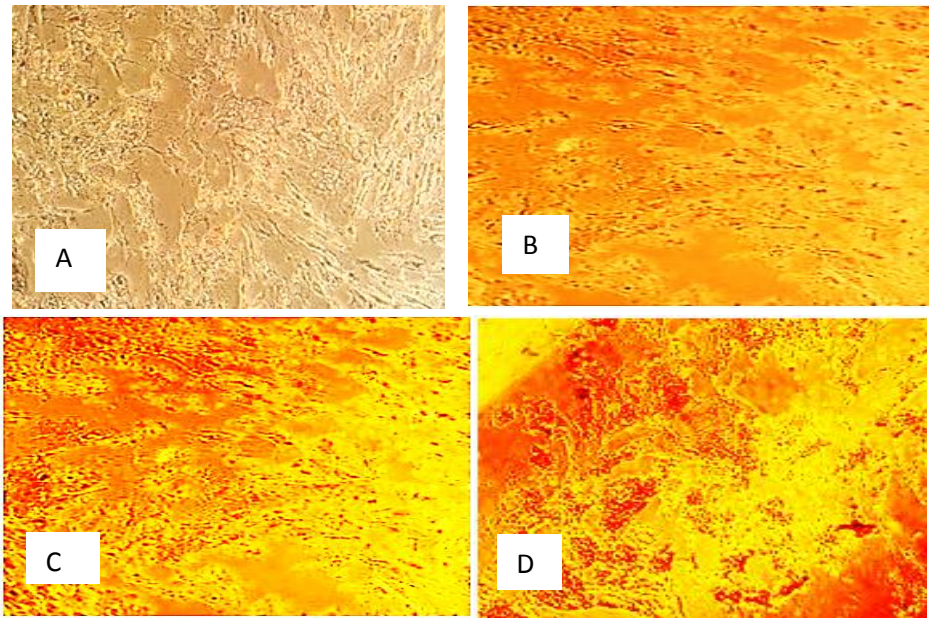

Figure 3: Figure shows Alizarin red staining. A: control, B: 800 µg/ml concentration OF saffron extract, B: concentration of 10^{-6} M vitamin D3. D: synergistic application 10^{-6} M vitamin D3and of 800 µg/ml Magnification (10×10).

Alkaline phosphatase (ALP) results:

Alkaline phosphatase test results (in the groups that was treated with Saffron): At plant extract concentrations of 600, 700 and 800 µg/ml ALP activity increased in 10 days compared to the control and a dose-dependent effect was observed in this group. At a same time point, 800 µg/ml of saffron extract showed the highest activity of ALP (Fig. 4).

Alkaline phosphatase test results (in the groups that were treated with vitamin D3): In the group that treated with vitamin D3, alkaline phosphatase activity showed a dose-dependent increase in the duration of 10 days. The most ALP activity was observed in 5×10^{-7} M of vitamin D3 (Fig. 4).

Alkaline phosphatase test results (in the groups that was treated with vitamin D3 and saffron extract): Concomitant use of vitamin and saffron lead to increased alkaline phosphatase activity in the treated group compared to the use of each of these factors alone (Fig. 4).

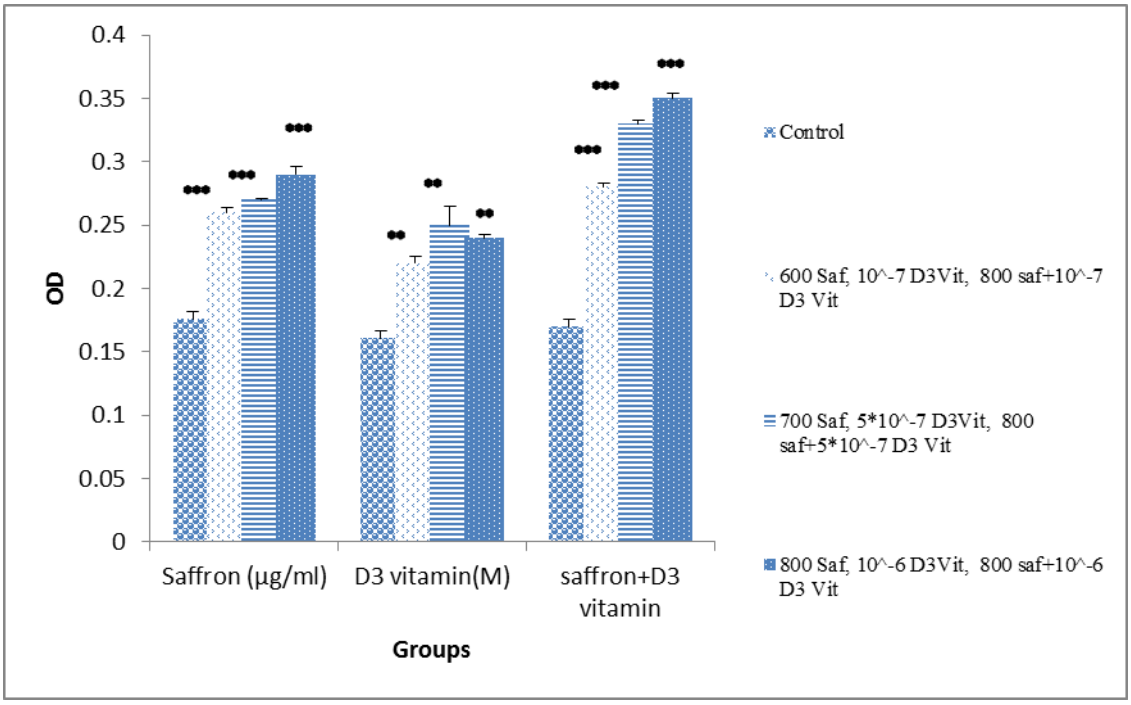

Figure 4: shows the activity of alkaline phosphatase at different doses of vitamin D3, saffron extract and synergies use of them (*** P < 0.001, ** P < 0.01., ANOVA, ments ± S.D)

RT-PCR results:

Osteicalcin, hall Markers for osteogenic differentiation, were assessed by comparative RT-PCR (Fig. 5). Significant upregulation of *osteocalcin* expreation take place in group that treatment with saffron extract and vitamin D3 together. *Osteocalcin* expression is associated with osteoblastic differentiation [17].

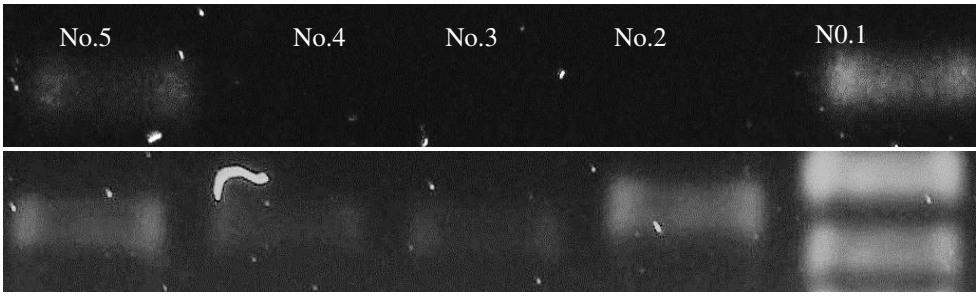

Figure 5: Agarose gel analysis of RT-PCR assays. The first lane No 1, represents molecular weight markers of *Hae*III restriction fragments of φX174 DNA. lane, NO 2. Control, lane No 3. vitamin D3, lane No 4.saffron extract, lane No 5. Synergist used of vitamin D3 and saffron extract. vitamin D3 and saffron extract RT-PCR analyses revealed that combined treatment with vitamin D3 and saffron extract induced early expression of osteocalcin mRNA on day 14, while treatment with vitamin D3 and saffron extract alone produced a relatively weak expression.

4 Discussion

Bone is composed of a mineralized matrix and bone cells. Osteoblasts are active mature bone cells that synthesized the organic matrix and regulate the mineralization process. Osteogenesis begins with osteoblast formation and secretion of type I collagen, which makes up about 90% of the organic bone matrix, or the osteoid [18] Once osteoblasts are active, they begin to produce huge amounts of ALP [19]. The proliferation rate and biological activity of the osteoblasts controls the rate of bone formation, and accelerated osteoblast growth is the key factor for efficient bone repair and osteoporosis. Bone BMSCs are the source of bone-forming osteoblasts [2]. As the result there is a need to identify factors that influence the differentiated stem cell to bone tissue specially blastocyst. This method is effective to improved abnormal conditions such as bone fractures and osteoporosis. As a result researcher has focused on finding new natural for induction osteoblast differentiation. Plants can provide alternative potential to this goal. In hence *we investigate the effects of extract from saffron and vitamin D3 on osteogenesis activity in, rat bone marrow mesenchymal stem cells.* Saffron consists of dried stigmas of the herb *Crocus sativus L.* It is cultivated not only in India and Iran but also in Mediterranean countries such as Greece, Spain and France [15] [18]. vitamin D3 is a secosteroid hormone that regulates bone formation [13]. *For this porpose, we examined cell cytotoxity of* saffron extract, vitamin D3 and combination of them. *This result showed that* BMSCs viability values after MTT assay were stable when treated with concentration below 10^{-7} M for vitamin D3 and concentration below from 400 µg/ml saffron extract compared to control group. This means there was no cytotoxicity when the concentration of vitamin D3 and saffron were lesser than this amount. But the OD values decreased when the concentration of vitamin D3 and saffron was larger than 10^{-6} M and 1000 µg/ml respectively. This means cytotoxicity increased when the concentration of the agents were more than this amounts. The result showed that the cytotoxicity of vitamin D3 and saffron extract limited the cell viability in 24h. Based the results from MTT assay we used vitamin D3 (10^{-7}, 5×10^{-7} and 10^{-6} M) doses and saffron extract (600,700 and 800 µg/ml) doses that are between lethal concentrations and ineffective concentration for induction differentiation. The cytotoxicity in Synergistic groups was evaluated, the results showed that the synergistic application saffron and vitamin D3 not only decrease cytotoxicity but also increase cell viability compared with the use of vitamin D3alone. This effect may bay due the protective effect of safranal, the active constituent of *Crocus sativus L.* stigmas in hence many research define the possible use of saffron as effective chemopreventive agent in clinical trials because this natural product show good protective affect agonist chemopreventive agent [20, 21]. To evaluate the osteogenic differentiation of stem cells that treated with saffron extract, vitamin D3 and synergistic application saffron and vitamin D3 the alkaline phosphatase activity and Alizarin red staining was used. ALP activity was determined by colorimetric assay which measures the enzymatic conversion of p-nitro-phenyl phosphate (p NPP) to the yellowish product p-nitro-phenol (pNP) in the presence of ALP.

The rate of pNP production is proportional to the amount of ALP in the sample solution [22]. ALP activity is one of the osteoblastic markers and has substantial function in mineralization. Our results showed that the saffron extract, vitamin D3and used them to gather Induced the ALP activity in contrast with the control group and the most enzyme activity was observed at 800 µg/ml saffron, 5×10^{7} M vitamin D3 and 800 µg/ml plant extract 10^{6} M vitamin D3 in combined group. This result inducing effects this observation is in agreement with a study which carried out on effect of Fennel extract [23]. Results from Alizarin red staining indicated the saffron extract also facilitated extracellular matrix mineralization, it increased in the presence of saffron extract, vitamin D3to gather specially in group that received 800 µg/ml plant extract $+10^{6}$ M vitamin D3. This corresponds with the results of alkaline phosphatase activity. Potu et al evaluate the effects of the *petroleum ether* extract of *Cissus quadrangularis* on the differentiation of marrow mesenchymal stem cells into osteoblasts (osteoblastogenesis) and extracellular matrix calcification. They results suggest that *Cissus quadrangularis* stimulates osteoblastogenesis and can be used as preventive/alternative natural medicine for bone diseases such as osteoporosis that is similar with our results [2].

Acknowledgment

This study was financially supported by the Research Center for Animal Development Applied Biology, Mashhad Branch Islamic Azad University Mashhad, Iran.

Reference

[1] D. Logeart-Avramoglou, f. Anagnostou, R. Bizios, H. Petite, Engineering bone: challenges and obstacles, J Cell Mol Med, 9 (2005) 72-84.
http://www.ncbi.nlm.nih.gov/pubmed/15784166

[2] B. K. Potu, K. M. Bhat, M. S. Rao, G. K. Nampurath, M. R. Chamallamudi, S. R. Nayak, M. S. MuttigiIV, Petroleum ether extract of cissus quadrangular is (linn.) enhances bone marrow mesenchymal stem cell proliferation and facilitates osteoblast genesis, Clinics, 64 (2009) 993-998.
http://dx.doi.org/10.1590/S1807-59322009001000010

[3] H. T Chen, B. M. JL, C. H. Chen, S. C. Chuang, L. F. Chang, M. L. Ho, S. H. Hung et al, Proliferation and differentiation potential of human adipose-derived mesenchymal stem cells isolated from elderly patients with osteoporotic fractures, J Cell Mol Med, 16 (2012) 582-592.
http://onlinelibrary.wiley.com/doi/10.1111/j.1582-4934.2011.01335.x/full

[4] J. J. Fan, L. G. Cao, T. Wu, D. X. Wang, D. Jin, S. Jiang, Z. Y. Zhang, L. Bi, G. X. Pei, The Dose-Effect of Icariin on the Proliferation and Osteogenic Differentiation of Human Bone Mesenchymal Stem Cells, 16 (2011) 10123-10133.
http://www.mdpi.com/1420-3049/16/12/10123

[5] N. Z. Mostafa, R. Fitzsimmons, P. W. Major, A. Adesida, N. Jomha, H. Jiang, H. Uluda, Osteogenic Differentiation of Human Mesenchymal Stem Cells Cultured with Dexamethasone, Vitamin D3, Basic Fibroblast Growth Factor, and Bone Morphogenetic Protein-2, Connective Tissue Research, 53 (2012) 117-131.
http://informahealthcare.com/doi/abs/10.3109/03008207.2011.611601

[6] Y. Yu, J. Mu, Z. Fan, G. Lei, M. Yan, S. Wang, C. Tang et al, Insulin-like growth factor 1 enhances the proliferation and osteogenic differentiation of human periodontal ligament stem cells via ERK and JNK MAPK pathways, Histochemistry and Cell Biology, 173 (2012) 513-525.
http://dx.doi.org/10.1007/s00418-011-0908-x

[7] A. Kumar, B. P. Salimath, G. B. Stark, G. Finkenzeller, Platelet-Derived Growth Factor Receptor Signaling Is Not Involved in Osteogenic Differentiation of Human Mesenchymal Stem Cells, Tissue Engineering, 16 (2010) 983-993.
http://dx.doi.org/10.1089/ten.tea.2009.0230

[8] E. Luzi, F. Marini, S. C. Sala, I. Tognarini, G. Galli, M. L. Brandi, Osteogenic Differentiation of Human Adipose Tissue-Derived Stem Cells Is Modulated by the miR-26a Targeting of the SMAD1 Transcription Factor, Journal of Bone and Mineral Research, 23 (2008) 287-295.
http://dx.doi.org/10.1359/jbmr.071011

[9] I. H. Kalfas, Principles of bone healing, Neurosurg Focus, 10 (2001) 1-4.
http://dx.doi.org/10.3171/foc.2001.10.4.2

[10] Z. L. Deng, K. A. Sharff, N. Tang, W. X. Song, J. Luo, X. Luo, J. Chen, E. Bennett, R. Reid, D. Manning et al, Regulation of osteogenic differentiation during skeletal development, Frontiers in Bioscience, 13 (2008) 2001-2021.
http://dx.doi.org/10.2741/2819

[11] A. Nakamura, Y. Dohi, M. Akahane, H. Ohgushi, H. Nakajima, H. Funaoka, Y. Takakura, Osteocalcin Secretion as an Early Marker of In Vitro Osteogenic Differentiation of Rat Mesenchymal Stem Cells, Tissue Engineering Part C: Methods, 15 (2007) 169-180.
http://dx.doi.org/10.1089/ten.tec.2007.0334

[12] Y. Feng, Y. Sun, W. Jia, C. Zhang, Platelet-rich plasma and 1,25(OH)2 vitamin D3 synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells, Biotechnology Letters, 32 (2010) 635-642.
http://dx.doi.org/10.1007/s10529-009-0198-8

[13] G. Penna, L. Adorini, 1a,25-Dihydroxyvitamin D3 Inhibits Differentiation, Maturation, Activation, and Survival of Dendritic Cells Leading to Impaired Alloreactive T Cell Activation, J Immunol, 164 (2000) 2405-2411.
http://dx.doi.org/10.4049/jimmunol.164.5.2405

[14] H. Hosseinzadeh, M. H. Modaghegh, Z. Saffari, Crocus Sativus L. (Saffron) Extract and its Active Constituents (Crocin and Safranal) on Ischemia-Reperfusion in Rat Skeletal Muscle, eCAM, 6 (2009) 343-350.
http://www.hindawi.com/journals/ecam/2009/146701/abs/

[15] C. Tzanidi, C. Proestos, P. Markaki, Saffron (Crocus sativus L.) Inhibits Aflatoxin B1 Production by Aspergillus parasiticus, Advances in Microbiology, 2 (2012) 310-316.
http://www.scirp.org/journal/PaperDownload.aspx?paperID=22711

[16] S. Sun, G. Chen, M. Xu, Y. Qiao, S. Zheng, Differentiation and Migration of Bone Marrow Mesenchymal Stem Cells Transplanted through the Spleen in Rats with Portal Hypertension, PLOS ONE, 8 (2013) 1-7.
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0083523#pone-0083523-g006

[17] D. M. Thomas, S. A. Carty, D. M. Piscopo, J. S. Lee, W. F. Wang, W. C. Forrester, P. W. Hinds, The Retinoblastoma Protein Acts as a Transcriptional Coactivator Required for Osteogenic Differentiation, Molecular Cell, (2001) 303-316.
http://dx.doi.org/10.1016/S1097-2765(01)00327-6

[18] S. M. Cool, Substrate Induction of Osteogenesis from Marrow-Derived Mesenchymal Precursors, Stem Cells and Development, 14 (2006) 632-642.
http://dx.doi.org/10.1089/scd.2005.14.632

[19] J. E. Compston, Principles of Bone Biology, Clinical Endocrinology, 46 (1997) 781.
http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2265.1997.2431019.x/abstract

[20] F. Abdullaev, J. Espinosa-Aguirre, Biomedical properties of saffron and its potential use in cancer therapy and chemoprevention trials, Cancer Detection and Prevention, 28 (2004) 426-432.
http://dx.doi.org/10.1016/j.cdp.2004.09.002

[21] H. Hosseinzadeh, H. R. Sadeghnia, Safranal, a constituent of Crocus sativus (saffron), attenuated cerebral ischemia induced oxidative damage in rat hippocampus, JPPS, 8 (2005) 394-399.
http://www.ualberta.ca/~csps/JPPS8(3)/H.Hosseinzadeh2/safranal.htm

[22] M. Stiehl, C. Bünger, A. Baatrup, M. Lind, M. Kassem, T. Mygind, Effect of dynamic 3-D culture on proliferation,distribution, and osteogenic differentiation of human mesenchymal stem cells, Wiley InterScience, 89A (2009) 96-107.
http://onlinelibrary.wiley.com/doi/10.1002/jbm.a.31967/abstract?deniedAccessCustomisedMessage=&userIsAuthenticated=false

[23] Z. Mahmoudi, M. Soleimani, A. Saidi, G. Khamisipour, A. Azizsoltani, Effects of Foeniculum vulgare ethanol extract on osteogenesis in human mecenchymal stem cells, Avicenna Journal of Phytomedicine, 3 (2013) 135-142.
http://ajp.mums.ac.ir/?_action=articleInfo&article=3&vol=5