Journal of Traditional and Complementary Medicine 12 (2022) 466–476

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Trans-differentiation of mouse mesenchymal stem cells into pancreatic β-like cells by a traditional anti-diabetic medicinal herb *Medicago sativa* L

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A R T I C L E I N F O

Article history:
Received 10 November 2021
Received in revised form
9 February 2022
Accepted 11 February 2022
Available online 21 February 2022

Keywords:
Medicinal herbs
Medicago sativa L.
Mesenchymal stem cells
Pancreatic β-cells
Trans-differentiation

A B S T R A C T

Background and aim: *Medicago sativa* L. is a medicinal herb first cultivated in ancient Iran. Traditionally, it has been utilized for the treatment of several disorders. The plant has been in the human diet for at least 1500 years. Although the hypoglycaemic and anti-diabetic effects of the plant have been approved in traditional medicine, further investigations are needed to support the rational use of *M. sativa* by humans. This project aimed to evaluate the *trans*-differentiation potential of bone marrow mesenchymal stem cells (MSCs) to pancreatic β-like cells (insulin-producing cells; IPCs) under the influence of *M. sativa* extract.

Experimental procedure: Bone marrow MSCs isolated, characterized, and then treated by flower or leaf extract of *M. sativa*. Beta-cell characteristics of the differentiated cells were evaluated by several techniques, including specific staining, QPCR, immunofluorescence, and ELISA.

Results: The results showed that the differentiated cells were able to express some specific pancreatic genes (PDX-1, insulin1, and insulin2) and proteins (insulin receptor beta, insulin, proinsulin, and C peptide). Furthermore, ELISA analysis indicated the ability of these cells in the production and secretion of insulin, after exposure to glucose.

Conclusion: Overall**,** both the flower and leaf extract of *M. sativa* had the potential of differentiation induction of MSCs into IPCs with the characteristics of pancreatic β–like cells. Therefore, *M. sativa*, as an herbal drug, may be beneficial for the treatment of diseases including diabetes.

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1. Introduction

Using natural herbs as non-pharmacological agents is an exciting approach to improve health. Several studies have shown that plant-based traditional medicine, combined with modern medicine, can manage various diseases, including diabetes.[1–4] Anti-cancer and anti-diabetic properties of some synthetic (vitamin C and vitamin E) and natural (saffron and its derivatives, crocin, safranal and crocetin, ginger, camel whey protein hydrolysates) products have been reported previously.[5–10] Herbal products, aescin (AES, a triterpene saponin), and diosmin (DIO, an unsaturated flavonoid glycoside) individually or in low-dose combination could ameliorate liver injury in rat hepatic damaged model.[11] Administration of single-celled green algae, chlorella, protected the pancreas of streptozotocin (STZ)-induced diabetic rats. This protective effect is probably due to the anti-hyperglycemic and antioxidant properties of the algae. In addition, treatment with chlorella in rats increased the proliferation and survival of pancreatic β-cells.[12] The potential of a medicinal herb, *Cichorium intybus* L., to induce *in vitro* differentiation of P19 embryonal carcinoma (EC) stem cells into pancreatic β-cells was reported in our recent study.[2]

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2022.02.002

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Differentiated cells expressed specific pancreatic genes and proteins and could also synthesize and release insulin in exposure to glucose. *Medicago sativa* Linn. (alfalfa, lucerne; a flowering plant in the Fabaceae family) is a traditional medicinal herb widely used in Iran, Turkey, the USA, India, Iraq, South Africa, and China. The plant was first cultivated in ancient Iran. Because of the diuretic effects of *M. sativa*, it has traditionally been used to treat urinary system disorders.[13] In addition, the anti-asthmatic, anti-arthritic, and anti-diabetic activities of alfalfa leaf have been previously proven in traditional medicine.[14] The protein- and vitamin-rich leaves of this plant have been used in the diet for at least 1500 years.[15] Adding alfalfa into diet and drinking water of STZ -diabetic mice reduced blood glucose.[16,17] Furthermore, BRIN-BD11 (a pancreatic β-cell line) released insulin in response to aqueous extract of the plant.[16] The cholesterol-lowering effect of alfalfa was confirmed by numerous studies in animals and humans. Aqueous extract of this medicinal herb reduced triglycerides, LDL (low-density lipoprotein), liver enzymes, and glucose in diabetic rats.[1] Alfalfa leaf extract and sprouts exert anti-hyperglycaemic and correct dyslipidemia, oxidative stress, and hepatic renal functions as effective as metformin (a standard hypoglycaemic drug for the treatment of type 2 diabetes) in diabetic rats.[18–20] Histological studies showed that the size and the number of pancreatic islets were increased after treatment of diabetic rats with this extract.[19] An *in vitro* study confirmed the anti-oxidant and enzyme inhibitory activity of ethanol extract of *M. sativa* .[21] Alfalfa leaf powder could reduce plasma glucose levels in patients with type II diabetes mellitus 2 h after ingestion. It also stimulates insulin secretion from the islets of healthy and diabetic people if taken with meal.[22] Despite the traditionally known hypoglycaemic activity of alfalfa both in animals and humans, so far, only a few investigations have been carried out to prove this issue. In the present study, *in vitro trans*-differentiation of mouse bone marrow mesenchymal stem cells (MSCs) into pancreatic β-like cells (insulin-producing cells; IPCs) under the influence of alfalfa flower and leaf extract was investigated for the first time. Type 1 diabetes (T1D) is caused by a deficiency of pancreatic β-cells. Replacement of destroyed cells with pancreatic functional β-cells, produced on a large scale from stem cells, can lead to long-term or even definitive treatment of the disease.

2. Materials and methods

2.1. Flavonoid extraction and quantification

Plant specimens of *Medicago sativa* L. were collected by the author(s) from Jahanbin mountain area (N 32.214, E 50.819, Alt.: 2033 m) in Bakhtiari province, one of the most diversity-rich areas in the Central Zagros of Iran. Specimens were identified by Fatemeh Hasanzadeh, at the herbarium of Shahrekord University, Shahrekord 115, Iran (voucher no. 4144). This project did not include a field study, and necessary permits for harvesting the plant have been obtained from Shahrekord University. This study with the plant *Medicago sativa* complies with local and national regulations.

The extraction and measurement of the plant flavonoids were carried out as described previously.[2] Briefly, methanolic solvents of dry plant materials (flowers and leaves) were incubated at 100 °C, dried in a rotary evaporator, and dissolved in warm distilled water (75 °C). Then, the cooled extracts were filtered by Whatman filter paper No. 1 to remove fatty substances and chlorophyll. After partitioning the extract by butanol, the upper flavonoid phase was collected. The dried extract was dissolved in distilled water, dried again, and dissolved in MeOH. By using a colorimetric method,[23] the total concentration of the flavonoids in flower (FE) and leaf (LE) extract was determined and the extracts were stored at 4 °C.

2.2. Analysis of the extract by HPLC

The extracts were analyzed by HPLC (model Agilent 1090). The HPLC elution method has been reported by Gharibi et al.[24] All standards (gallic acid, chlorogenic acid, caffeic acid, syringic acid, rutin, luteolin-7-O-glucoside, 1,3 dicaffeoylquinic acid, rosmarinic acid, Luteolin, quercetin, apigenin, and chicoric acid) were dissolved in HPLC grade methanol before injection to the analytical HPLC system. For analysis, A 0.22 μm nylon acro-disk filter and 20 μL of the extract were used for injection. The stationary phase possessed 250 mm × 4.6 mm (5 μm) symmetry C18 column (Waters Crop., Milford, MA, USA) (10 mm × 4 mm I.D.). The mobile one included 0.1% formic acid in acetonitrile (flow rate of 0.8 mL min^{-1}) with the wavelength between 200 and 400 nm. The column was 25 °C. 0.1% of water-formic acid was applied as solvent A, while 0.1% of B for 70 min, and finally to 100% solvent B for 75 min. The polyphenolic compounds were evaluated by comparing the peak areas and retention times. Finally, the results were reported as mg/100 g of the sample dry weight.

2.3. Isolation, expansion, and identification of MSCs

BALB/c mouse strain was purchased from Azad University of Shahrekord (Shahrekord, Iran) and kept under standard housing conditions. All of the animal procedures in the present study were performed according to the rules and regulations set by the Bioethics Committee of the University of Isfahan, based on the National Specific Ethical Guidelines for Biomedical Research issued by the Ministry of Health and Medicinal Education (MOHME) of Iran in 2005. The protocols were performed under the relevant guidelines and regulations. The experimental protocols were approved by MOHME. The isolation of mouse bone marrow MSCs was carried out as described previously.[25] Briefly, under deep anesthesia the femur and tibia of 2-month-old male mice were perfused, and the resulted bone marrow cells were cultured in DMEM (Dulbecco's modified Eagle medium; Gibco, 31600), 10% FBS (fetal bovine serum, Gibco, 10270-106), 50 μg/mL penicillin (Sigma, P3032), and 50 μg/mL streptomycin (Sigma, S1277). After 24 h, the non-adherent hematopoietic cells were discarded, and the cultures were passaged at 80% confluency. To determine the multipotency characteristics of MSCs the cells were exposed to osteogenic or adipogenic induction media The differentiation potential was then confirmed by alizarin red and oil red staining, respectively.[25] Previously, the “multipotency” of MSCs has been further confirmed using flow cytometry in our recent report.[26]

2.4. Viability and proliferation assay

Cell viability and proliferation of control, FE-, and LE-treated groups were performed at 24, 72, and 96 h by a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma, M5655) assay.[27] The absorbance was measured at 570 nm, and the mean values from at least three repeats were calculated.

2.5. Trans-differentiation induction of MSCs

Three experimental groups were designed as follows: 1) FE: MSCs treated by flower extract of *M. sativa* (DMEM + 5% FBS + 50 μg/ml FE); 2) LE: MSCs treated by leaf extract of *M. sativa* (DMEM + 5% FBS + 50 μg/ml LE); and 3) Cont: MSCs with no treatment as control group (DMEM + 5% FBS). To induce differentiation, MSCs were cultured in gelatin-coated dishes at passages 3–5. The cells were then exposed to 50 μg/ml of FE or LE, and the induction media were replaced after 24 hr[2]. The differentiation potential of the treated MSCs was evaluated after about 12 days, with different methods. A

control group without any treatment was also considered. All the experiments were repeated at least three times.

2.6. DTZ staining

The initial characterization of IPCs was evaluated by DTZ (dithizone; diphenylthiocarbozone, Merck, DX2370-3) staining.[28] Briefly, the stock DTZ solution was directly added to the medium. After incubation for 15–30 min at 37 °C, the cells were rinsed with hank's balanced salt solution (HBSS), and the DTZ-positive (DTZ^+) cell clusters were detected by stereomicroscope. To estimate the percentage of DTZ^+ cells, the cultures were trypsinized, and the single crimson red cells were counted at 40X magnification using a Neubauer.[29]

2.7. RNA extraction and QPCR

QPCR was utilized to quantify the relative expression level of the genes,[29] including insulin1, insulin2, PDX-1 (pancreatic and duodenal homeobox 1), EP300 (E1A binding protein p300), and CREB1 (cAMP-responsive element binding protein 1) by specific primers (Supplementary materials, Table 1) and SYBR Premix Ex Taq (Takara, RR081Q). Also, a housekeeping gene, β-2M (β-*2* microglobulin), was considered as the internal control. Total RNA was extracted by Qiazol lysis reagent (Qiagen, 79306), and cDNA was synthesized by PrimeScript™ RT reagent Kit (Takara cDNA kit, RR037A_e.v1112Da). RT–PCR assay was performed by StepOnePlus™ Real-Time PCR System. All the samples, including untreated and treated groups and negative controls (no template or no primers), were run together. The standard curves were designed for each gene to analyze the efficiency of the reactions. After cDNA replication, the Ct values were imported into Microsoft Excel and normalized in relation to the individual β-2M gene. The profile was obtained by plotting the relative gene expression levels compared to untreated control cells.

2.8. Immunostaining

For immunostaining,[29] the undifferentiated and differentiated cells were fixed and permeabilized. Normal goat serum (NGS, Sigma, G9023) was used to block the sites of nonspecific binding of primary antibodies. The primary antibodies in the present study were mouse monoclonal proinsulin + insulin (Abcam, ab8304-50), rabbit polyclonal anti-C peptide (Abcam, ab14181), and mouse monoclonal insulin receptor beta (Abcam, ab8304-100). Cy5.29-conjugated anti-rabbit IgG (Abcam, ab6564) and FITC-conjugated anti-mouse IgG (Sigma, F9137) were applied as secondary antibodies. The nuclei were counterstained by DAPI.

2.9. Assessment of insulin production and secretion by ELISA

To measure intracellular and secretory insulin levels without fetal serum intervention,[29] the culture medium was replaced by a fresh FBS-free medium containing 0.5% BSA (bovine serum albumin). For the glucose challenge, low and high glucose (5.5 and 25 mmol/l) media were sequentially added to the cells. Enzyme-linked immunosorbent assay (ELISA) was performed on conditioned medium, and cell extract by insulin mouse ultrasensitive ELISA kit (Alpco, 80-insmsu-E01). The values were normalized based on the total protein.

2.10. Statistical analysis

At least three repetitions were considered for each experiment. Data were analyzed according to the GLM procedure of SAS statistical software (version 8; SAS Institute Inc., Cary, NC, United States). Statistical differences between the mean values were compared using Student's t-test for two groups and ANOVA (one-way analysis of variance) and LSD (least significant difference) test for more than two groups. The values were reported as mean ± SD (standard deviation), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical analysis

Phytochemical analysis showed that the concentration of total flavonoids was very different in the two extracts (FE; 16.262 mg/ml, LE; 34.180 mg/ml). The results of the HPLC analysis are illustrated in Table 2 (Supplementary materials, Table 2). Rosmarinic acid, apigenin, syringic acid, and 1,3 dicaffeoylquinic acid were the major components of *Medicago sativa* methanolic extract. Apigenin and rosmarinic acid showed the highest rates in *Medicago* leaf and flower, respectively. The concentration of some phenolic compounds in FE was higher than their concentration in LE, which could be the reason for the higher effects of flower extracts, as shown by the other analysis reported here.

3.2. Isolation, expansion, and identification of MSCs

Bone marrow cells isolated from mouse femur and tibia were cultured and expanded to passage five (Fig. 1A). On the first day of culture (passage 0; P0), a percentage of spherical cells gradually began to adhere. Non-adherent cells were removed after 24 h. The attached spindle-shaped cells were sub-cultured at 70–80% confluency. The representative photomicrographs of the cells with fibroblast-like morphology have been shown in passage 3 (P3) and 5 (P5) in Fig. 1A. About the 15th day of culture, the homogeneous populations of MSCs tended to form distinct cell clusters in passage 5. Furthermore, osteogenic (Fig. 1B) and adipogenic (Fig. 1C) differentiation of MSCs confirmed their multipotency characteristics.

3.3. Cell viability assessment

The evaluation of cell viability was carried out by MTT colorimetric assay on experimental; FE- and LE-treated, and untreated groups (Fig. 2). The results showed that although after 24, 72 and, 96 h, there were no significant differences between some groups, however, the other groups showed statistically significant differences. After 24 h, the cell number of the FE-treated group (174.049 ± 8.226, $p > 0.05$) was at the highest level compared to that of the LE-treated (125.949 ± 2.777, $p > 0.05$) and control (131.622 ± 7.909, $p > 0.05$) groups. Furthermore, the number of the cells treated by FE (209.939 ± 23.997, $p > 0.05$) was in a satisfactory state after 96 h of culture. It showed no significant difference compared to the untreated group (230.659 ± 17.025, $p > 0.05$). Overall, MTT analysis confirmed that all groups were in an acceptable status regarding cell viability and proliferation.

3.4. Initial characterization of MSCs-derived IPCs

To evaluate the morphological features of pancreatic β-cells of the differentiated cells, DTZ specific staining was performed on all treatment and control groups (Fig. 3). Dithizone, as a zinc-chelating agent, can bind zinc ions in pancreatic islet's β-cells. Here, the individual cells and the islet-like cell clusters stained crimson red by the dye. The positive reaction to the stain was obviously visible in both FE- and LE-treated MSCs. Undifferentiated spindle-shaped cells and the cells in the control group didn't show a positive

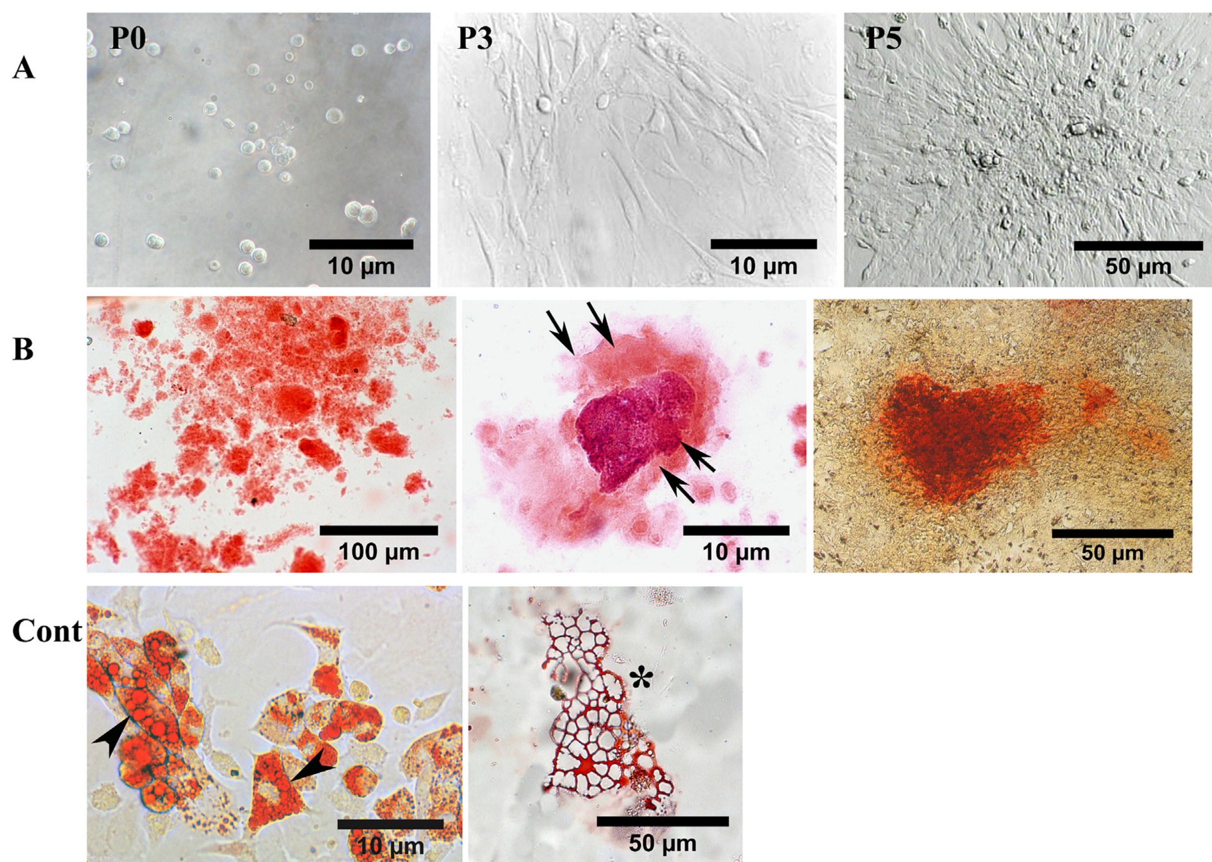

Fig. 1. Isolation, expansion, and identification of bone marrow mesenchymal stem cells (MSCs). (A) Mouse MSCs isolated from femurs and tibia and cultured in appropriate condition (P0: Passage 0). Attached spindle-shaped MSCs at passage 3 (P3) and a cell aggregate at passage 5 (P5) have also been presented. (B) Osteogenic differentiation of MSCs confirmed the multipotency characterization of the cells. Arrows show osteoblast-like cells with an epithelial arrangement. (C) Adipogenic differentiation of MSCs confirmed the multipotency characterization of the cells. The differentiated cells showed a morphology similar to brown, multilocular (Arrowheads), or white, unilocular (Asterisk) adipose tissue.

response to DTZ. Counting trypsinized individual cells indicated that the number of DTZ$^+$ cells in both experimental groups was significantly higher than that of the control group (FE: 28.715 ± 4.608, LE: 41.987 ± 9.697, C: 8.496 ± 5.866, $p > 0.05$). However, there was no significant difference between FE- and LE-treated groups.

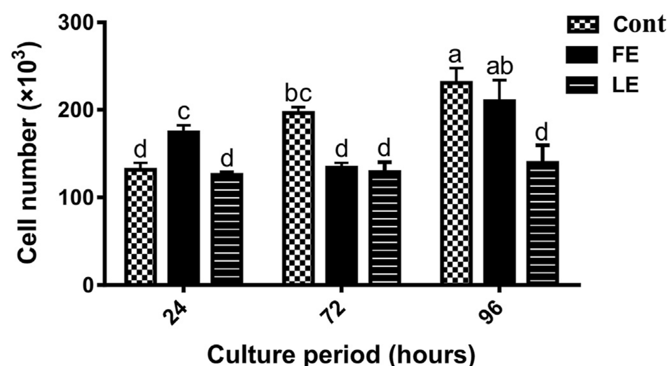

Fig. 2. MTT analysis of mesenchymal stem cells (MSCs), treated by plant extract. The results showed that although after 24, 72 and, 96 h, there were no significant differences between some groups, however, other groups showed statistically significant differences. After 24 h of culture, the highest cell number was achieved by the FE-treated group compared to those of the other two groups. There was no significant difference between control and the cells treated with LE after 96 h of treatment. The experiments were carried out at least in triplicate. The bars represent the standard error and the values (mean ± SD) with different lowercase letters indicate significant differences by the LSD test at $P < 0.05$. For variables with the same letters, the difference is not statistically significant, and for variables with different letters, the difference is statistically significant. Cont: control, FE: flower extract, LE: leaf extract.

3.5. Analysis of specific pancreatic gene expression induction

QPCR was applied to analyze the expression induction of specific pancreatic genes, PDX-1, insulin1, and insulin2, in the differentiated cells (Fig. 4A). Although the expression of the PDX-1 gene in both treated groups (FE: 5.063 ± 0.093, LE: 5.871 ± 1.055, $p > 0.05$) was significantly higher than that of control, there was no significant difference between those of FE- and LE-treated cells. The expression of insulin1 (FE: 7.845 ± 1.857, LE: 2.893±, 0.974, $p > 0.05$) and insulin2 (FE: 5.718 ± 0.597, LE: 2.327 ± 0.235, $p > 0.05$) in both induction media was significantly more than that of the control group, and the significant highest level belonged to FE-treated cells. The expression of transcription factors EP300 and CREB1 also was analyzed by QPCR (Fig. 4B). The results indicated that both extracts were able to induce EP300 gene expression in the differentiated cells with no significant difference (FE: 5.318 ± 0.228, LE: 6.126 ± 0.801, $p > 0.05$). Furthermore, the cells treated by LE showed significant CREB1 gene expression (17.804 ± 2.738, $p > 0.05$). However, the expression of CREB1 in FE-treated cells (0.911 ± 0.057, $p > 0.05$) was significantly lower than that of LE-treated and control cells.

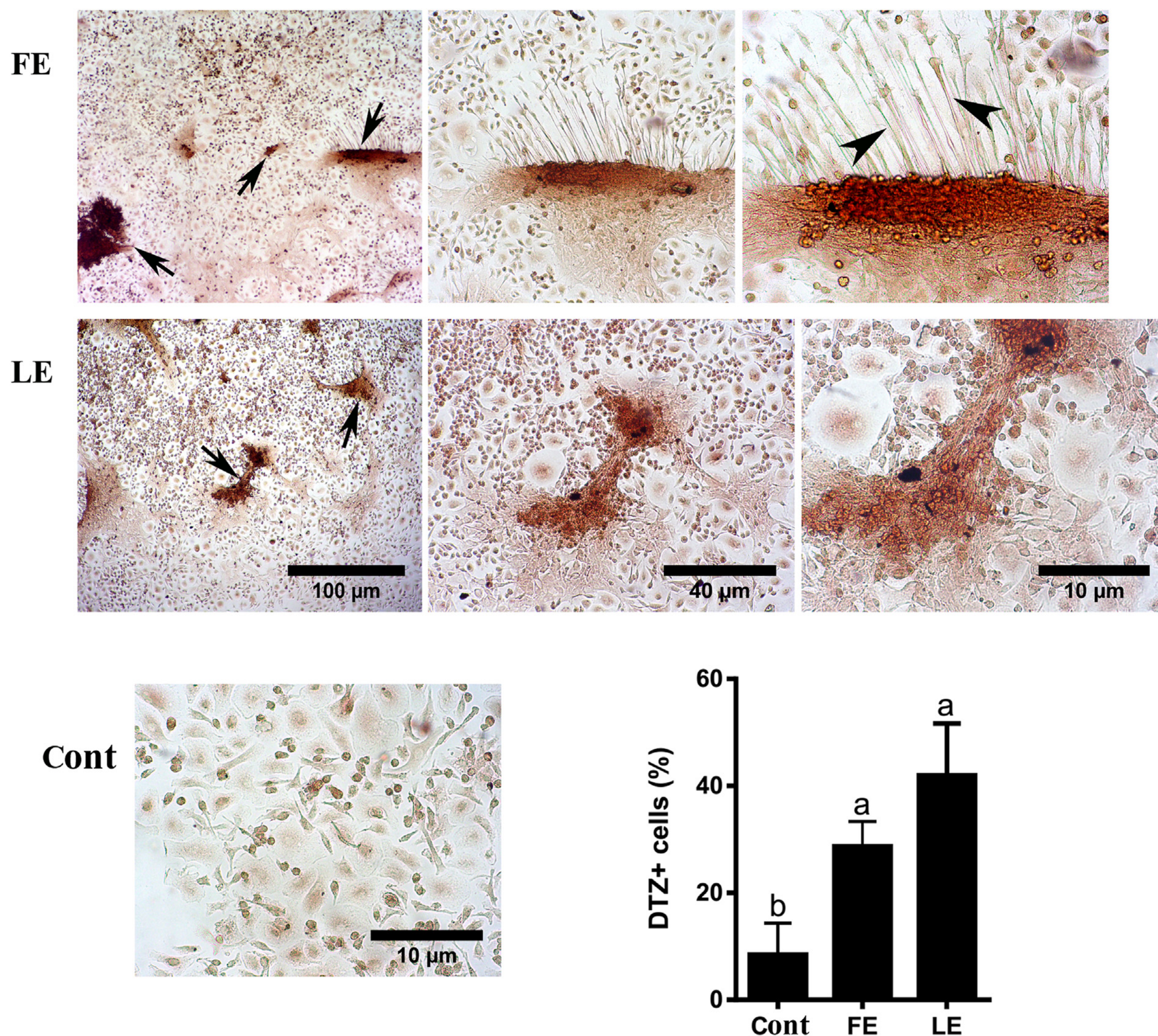

Fig. 3. Dithizone (DTZ) staining for the initial characterization of insulin-producing cells (IPCs). Mesenchymal stem cells (MSCs) treated by plant extract were differentiated and produced DTZ-positive (DTZ^+) cell aggregates (Arrows). Spindle-shaped MSCs (Arrowheads) showed no positive response to DTZ. There were a few sporadic individual DTZ^+ cells in the untreated control group. The graph indicates that the percentage of DTZ^+ cells in both treated groups was significantly more than that of the control group. The experiments were carried out at least in triplicate. The bars represent the standard error and the values (mean ± SD) with different lowercase letters indicate significant differences by the LSD test at $P < 0.05$. For variables with the same letters, the difference is not statistically significant, and for variables with different letters, the difference is statistically significant. Cont: control, FE: flower extract, LE: leaf extract.

3.6. Analysis of specific pancreatic protein expression induction

Immunostaining by antibody against nestin (Nes) demonstrated that the differentiated cells expressed this intermediate filament which is a marker for pancreatic islet precursors and neuro-epithelial cells (Fig. 5). In contrast, untreated control cells show no significant fluorescence reaction to this antibody. Double-immunostaining of the differentiated cells with specific antibodies confirmed that the cells treated by FE and LE were immunoreactive to pancreatic β-cell specific proteins, insulin + proinsulin (Ins + Pro), and C peptide (CP) (Fig. 6) as well as insulin receptor-β (Recβ) and C peptide (Fig. 7). However, untreated control cells didn't show an apparent antibody reaction.

3.7. Evaluation of insulin production and secretion by IPCs

The ability of insulin synthesis and secretion of MSCs-derived IPCs in response to glucose challenge was determined by ELISA (Fig. 8). Compared to LE-treated and control groups (LE: 0.781 ± 0.030, C: 0.603 ± 0.149, $p > 0.05$), the cells exposed to FE were significantly able to synthesize the most level of insulin (1.137 ± 0.020, $p > 0.05$) in response to 25 mM glucose (Fig. 8A). The significantly higher level of secreted versus intracellular (0.764 ± 0.234, $p > 0.05$) insulin in FE-treated cells indicated that the differentiated cells not only synthesized insulin, but also they were functional and able to secret the hormone in response to 25 mM glucose (Fig. 8B).

4. Discussion

In the current study, the differentiation potential of MSCs into pancreatic β–like cells was investigated. Flower and leaf extracts of a traditional anti-diabetic medicinal herb, alfalfa, were used to induce differentiation. The isolated MSC cells were first characterized and then exposed to 50 μg/ml of each extract.[2] Initial identification of obtained IPCs was confirmed by DTZ specific staining.

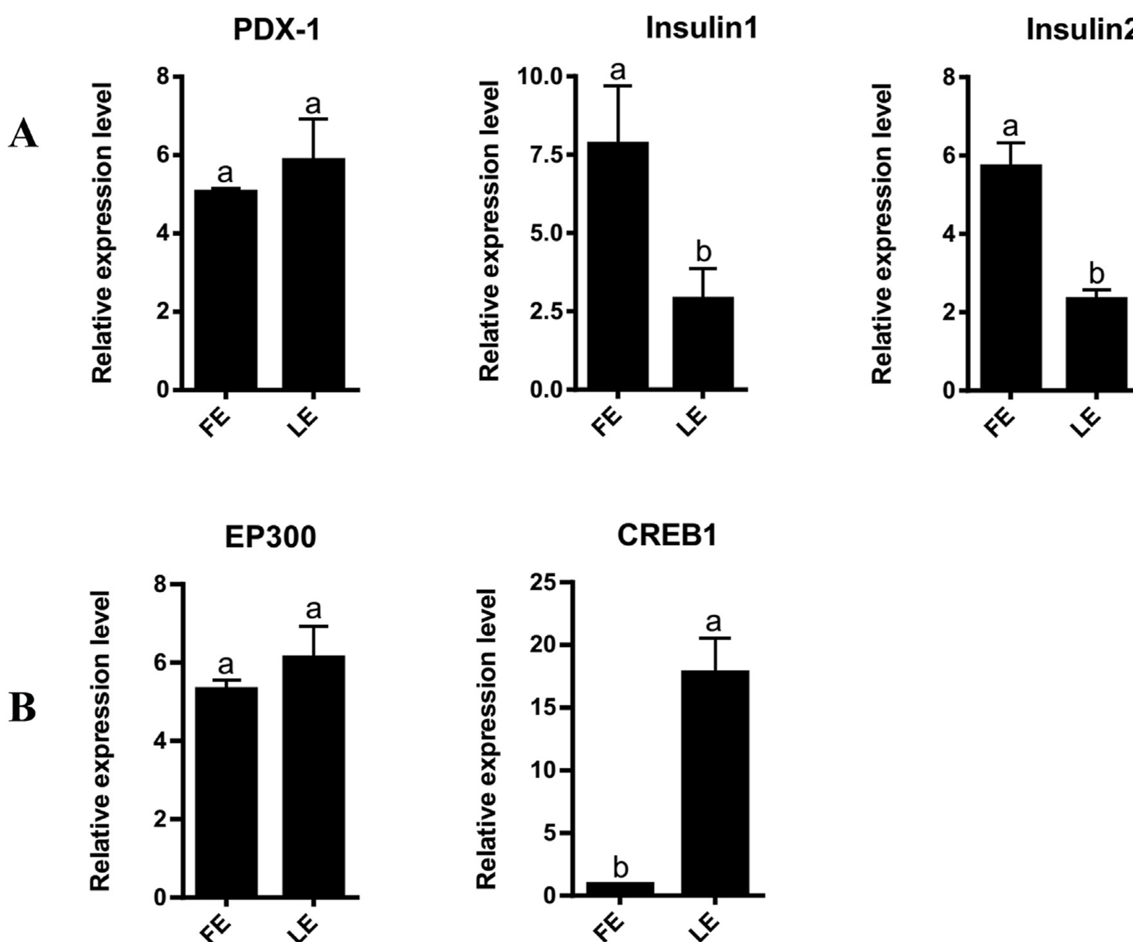

Fig. 4. Analysis of specific pancreatic gene expression induction by QPCR. (A) The expression of PDX-1 gene in both treated groups was significantly higher than that of the control group. There was no significant difference between those of FE- and LE-treated cells. Insulin1 gene expression in both induction media was considerably more than that of the control group, and the significant highest level belonged to FE-treated cells. (B) The expression analysis of transcription factors EP300 and CREB1 indicated that both extracts were able to induce EP300 gene expression in the differentiated cells with no significant difference. Furthermore, the cells treated by LE showed significant CREB1 gene expression. However, the expression of CREB1 in FE-treated cells was significantly lower than that of LE-treated and control cells. The experiments were carried out at least in triplicate. The bars represent the standard error and the values (mean ± SD) with different lowercase letters indicate significant differences by the LSD test at $P < 0.05$. For variables with the same letters, the difference is not statistically significant, and for variables with different letters, the difference is statistically significant.

The expression of some specific pancreatic genes and proteins was demonstrated by QPCR and immunofluorescence techniques, respectively. ELISA analysis indicated the ability of the differentiated cells to produce and release insulin in response to different glucose challenges.

Previous studies have confirmed the dramatic effect of some animal tissue extracts/conditioned media on stem cell differentiation. Recently, the effect of neonatal rat brain extract (NRBE) on neural differentiation of an EC stem cell line, P19, was approved.[30,31] Furthermore, the effects of neonate mouse pancreas extract (MPE)/conditioned medium (PCM) on the differentiation of P19 cells into pancreatic β-cells have been demonstrated previously.[29,32] MPE was also able to induce *trans*-differentiation of bone marrow mesenchymal stem cells into IPCs.[26] It seems that tissue-specific microenvironments are able to direct stem cells into a special lineage.[33] However, despite their non-specificity, the extracts of plants such as medicinal herbs induce the differentiation of stem cells toward specific lineages. So far, osteogenic,[34] chondrogenic,[35] neurogenic[36] differentiation of stem cells, and stimulation of stem cell proliferation[35,37] by various herbal extracts have been proven. In our recent work, *in vitro* differentiation of P19 EC cells into insulin-producing cells by *C. intybus* L. leaf extract was reported.[2]

MSCs can be isolated from a variety of sources, including bone marrow (BM), adipose tissue (AT), umbilical cord (UC), dental pulp, periodontal ligament, tendon, skin, muscle, and other tissues. Given that MSCs derived from different sources have different therapeutic potentials, which cell source is more appropriate and effective in clinical applications is still controversial. The most well-known and widely used source of MSCs is BM.[38] However, obtaining MSCs from some tissues such as BM and AT is an invasive procedure. Jin et al. (2013) claimed that because UC-MSCs have biological advantages over adult cell sources, these cells are useful model for cell therapy.[39] Although MSCs isolation from UC is a non-invasive method, the use of this source has been doubted by some researchers due to their low isolation efficiency.[38] On the other hand, multiple units of stem cells can be collected from BM, whereas only one UC unit is available from each donor. AT and BM are the most widely used sources of MSCs, due to their ease of harvesting and potential autologous application.[40] The advantages of MSCs (anti-inflammatory, immunomodulatory, high differentiation potential, paracrine ability) make their application in regenerative medicine promising. However, some undesirable properties of these cells (heterogeneity, potential tumorigenicity, potential side effects of transplanted cells, and severe donor age requirements) limit their use in tissue repair.[39,41,42] Overall, many biological advantages of various MSCs should be

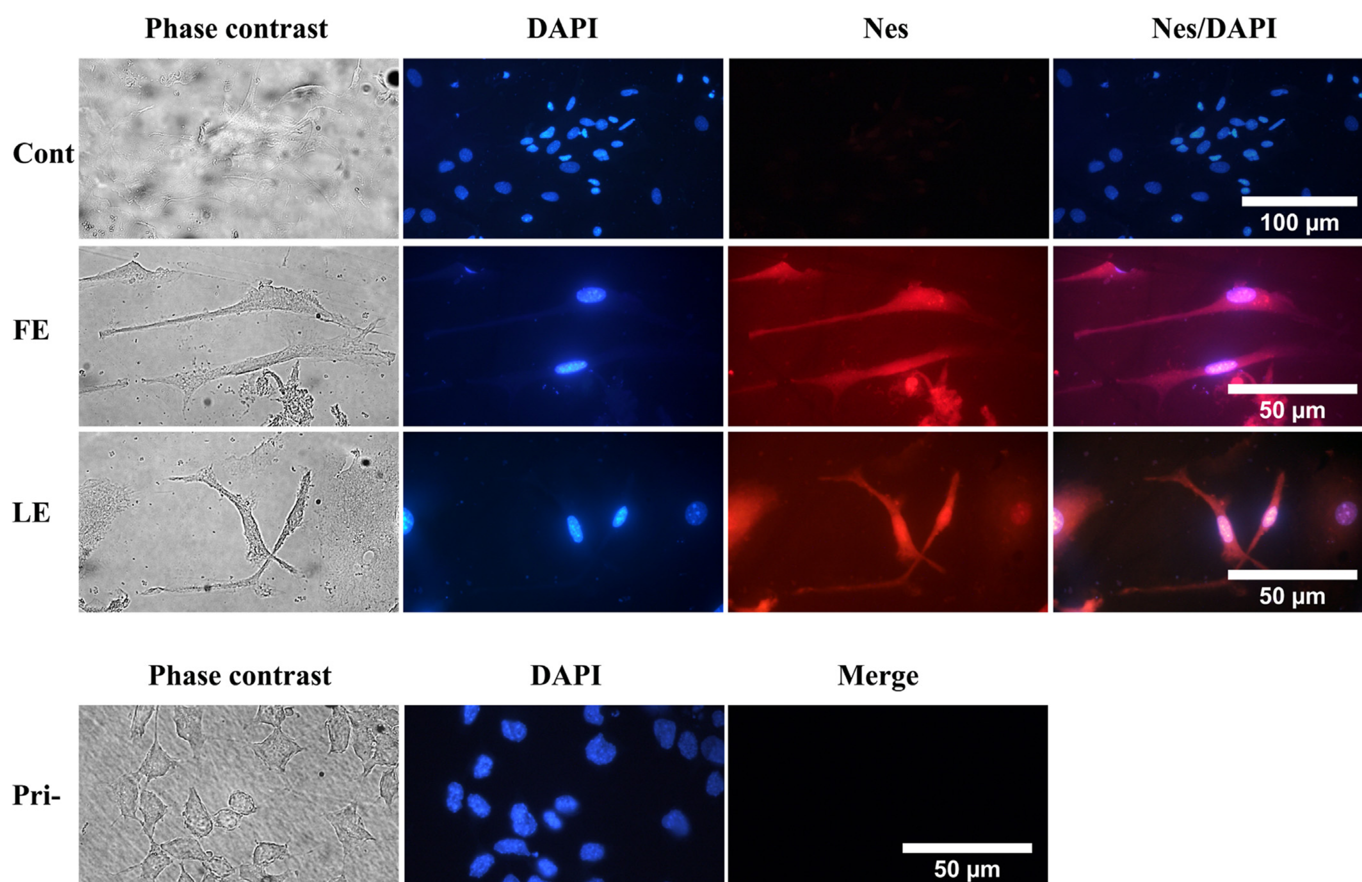

Fig. 5. Representative fluorescence micrographs for nestin (Nes), an intermediate filament presented in pancreatic islet precursors and neuroepithelial cells. The results show the expression of nestin (red) in both experimental groups; the cells treated by flower (FE), and leaf (LE) extract. There was no significant fluorescence reaction in the untreated control group (Cont). The nuclei were counterstained by DAPI (blue). Also, a merged image of nestin and DAPI (Nes/DAPI) was presented. A group without primary antibodies (Pri$^-$) was considered as the control for immunostaining.

systematically considered when selecting the cell source for specific clinical applications. In the present study, the *trans*-differentiation of a type of animal adult stem cells, MSCs, was induced using alfalfa extract as an anti-diabetic medicinal herb. *Trans*-differentiation is a process in which a committed cell transforms into a different cell type of another lineage through genetic reprogramming. Adult stem cells are committed progenitors with limited plasticity that can only differentiate into tissue-specific cell types.[43] Unlike committed cells, naive stem cells do not express baseline levels of lineage-specific markers. Despite their low plasticity, MSCs -as expected from multipotent stem cells-are able to respond dramatically to their microenvironment and undergo differentiation.[44] Human mesenchymal stem cells (hMSCs) that have been pre-committed to one mesenchyme cell lineage could *trans*-differentiate into other cell types in response to inductive extracellular cues.[45] Fully differentiated adipocytes-, chondrocytes-, and osteocytes-derived from MSCs could cross differentiation boundaries and *trans*-differentiate into other cell types.[45] The neuronal *trans*-differentiation potential of hMSCs has been demonstrated in several recent reports.[46,47] Furthermore, there are some examples of functional *trans*-differentiation of liver cells[48] and MSCs[49–52] into IPCs. Given the differentiation potential of MSCs into a different cell type from another lineage, here in this project, we used the word "*trans*-differentiation" for IPCs produced in our culture system.

The results clearly confirmed that the differentiated cells showed some characteristics of mature functional pancreatic β-cells. For instance, the FE- and LE-treated cells expressed some pancreas-specific markers such as PDX-1. This transcription factor (also known as IPF-1; insulin promoter factor-1) is the earliest marker for the differentiation of pancreatic cells.[53] It plays a crucial role in pancreas development, β-cell maturation and survival, and regulation of insulin gene expression. Furthermore, IPCs produced here expressed two mature forms of insulin: insulin1 & 2. In addition to proinsulin, these cells could also express C-peptide, which indicates their ability to synthesize and process insulin. C-peptide is a product of proinsulin breakdown, released by β-cells into the bloodstream.[54] The expression of proinsulin is one of the main features of mature pancreatic β-cells.[55] All factors mentioned above, are among the prominent features of mature pancreatic β-cells. However, for accurate production and identification of insulin-secreting cells, the authors recommend evaluating factors such as glucagon, somatostatin, PDX-1, and Nkx6.1 using immunostaining and flow cytometry. Co-expression of insulin, PDX1, and NKX6.1 (insulin+/PDX1+/NKX6.1+) is among the reliable features of mature functional β-cells.[56] NKX6.1 is a transcription factor that plays a critical role in pancreatic β_cell development. In adult islets, NKX6.1 is expressed exclusively in β-cells.[56,57] Interestingly, both PDX1 and Nkx6.1 inhibit transcription of glucagon gene.[58,59] Some investigators introduced a combined 3D (three-dimensional) protocol using PVA (polyvinyl alcohol) scaffold and PRP (platelet-rich plasma) or poly-L-lactic acid/polyvinyl alcohol (PLLA/PVA) nanofibers that enhanced differentiation of hADSCs (human adipose-derived from mesenchymal stem cells) into IPCs compared to 2D (two-dimensional) culture.[60,61] Although our results confirmed

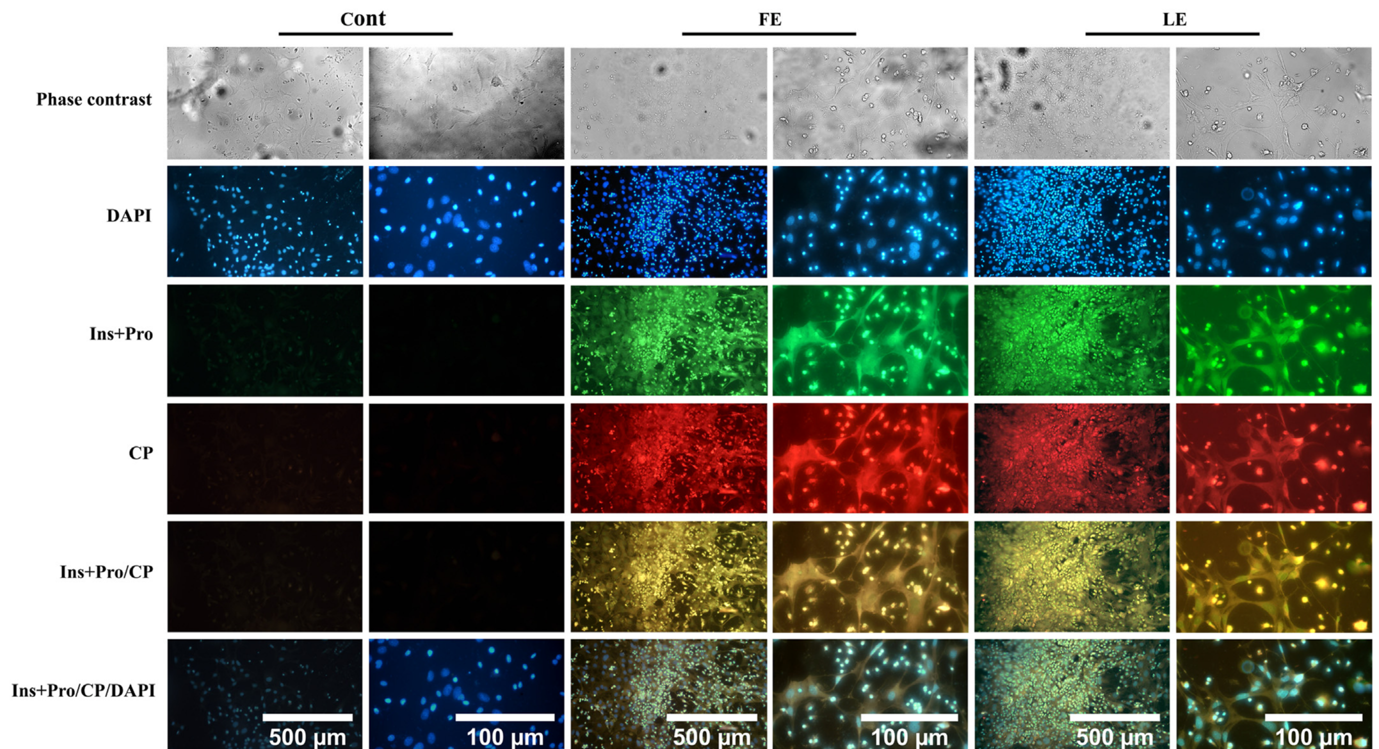

Fig. 6. Double-immunostaining of the differentiated cells with specific antibodies showed that the cells treated by flower (FE) and leaf (LE) extract were immunoreactive to pancreatic β-cells specific proteins, insulin + proinsulin (Ins + Pro, green), and C peptide (CP, red). Merged images (Ins + Pro/CP) indicated the simultaneous expression of both proteins in the differentiated cells. Untreated control cells (Cont) didn't show a significant reaction to the antibodies. Representative fluorescence micrographs were presented at low and high magnifications in each case. The nuclei were counterstained by DAPI (blue).

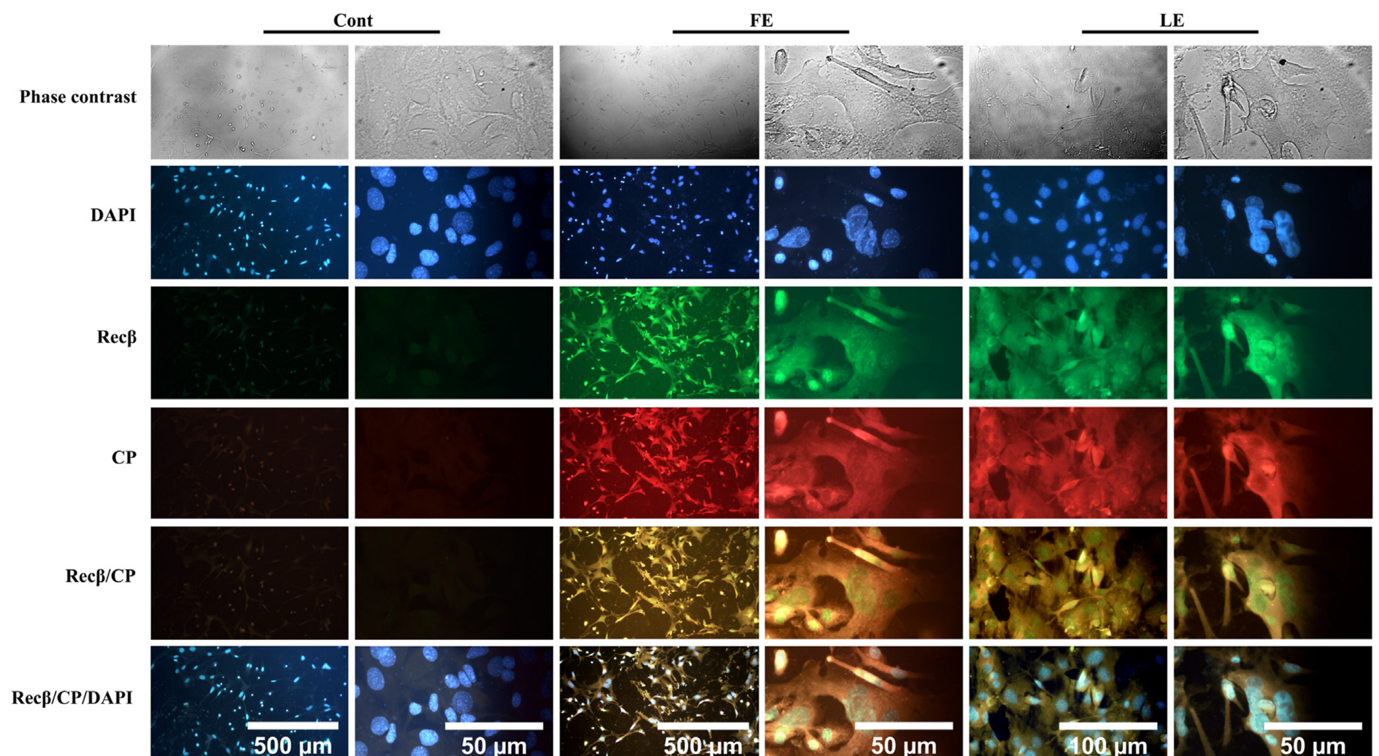

Fig. 7. Double-immunostaining of the differentiated cells with specific antibodies showed that the cells treated by flower (FE) and leaf (LE) extract were immunoreactive to pancreatic β-cells specific proteins, insulin receptor-β (Recβ, green), and C peptide (CP, red). Merge images (Ins + Pro/CP) indicated the simultaneous expression of both proteins in the differentiated cells. Untreated control cells (Cont) didn't show a significant reaction to the antibodies. Representative fluorescence micrographs were presented at low and high magnifications in each case. The nuclei were counterstained by DAPI (blue).

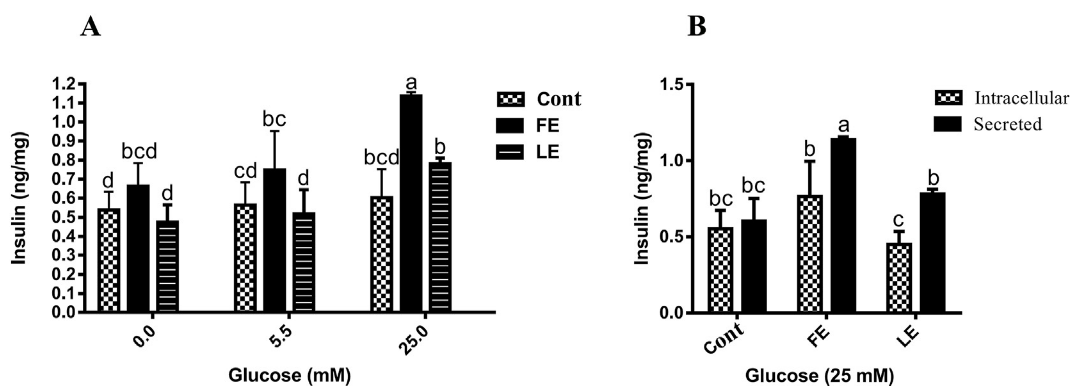

Fig. 8. Evaluation of glucose-dependent insulin production and secretion by the differentiated cells with different glucose challenges (low and high; 5.5 and 25 mmol/l). (A) Significant enhancement of insulin production was observed in FE-treated cells at 5.5 and 25 mM glucose. (B) Insulin secretion by FE-treated cells was significantly more than that of the other groups. The experiments were carried out at least in triplicate. The bars represent the standard error and the values (mean ± SD) with different lowercase letters indicate significant differences by the LSD test at $P < 0.05$. For variables with the same letters, the difference is not statistically significant, and for variables with different letters, the difference is statistically significant. Cont: control, FE: flower extract, LE: leaf extract.

producing mature functional pancreatic β-cells, using a 3D differentiation system which mimics the extracellular matrix, might even improve the resulting IPCs.

Traditionally, alfalfa leaves have been used for the treatment of diabetes.[62] A human diet containing alfalfa can improve HDL (high-density lipoprotein) levels while lowering triglycerides, LDL, and plasma glucose.[63] Consumption of aqueous alfalfa extract by diabetic rats reduced blood glucose level and enhanced the diameter of pancreas Langerhans islets.[1] Overall, it has been shown that alfalfa stimulates insulin secretion and improves its function in reducing blood glucose. However, its effects on *in vitro* differentiation and function of stem cells have not yet been addressed. MSCs-derived IPCs in the present study were able to produce and secret insulin in response to glucose challenges. Consistent with this view, incubation of BRIN-BD11 pancreatic B-cell line with alfalfa extract resulted in dose-dependent insulin secretion.[16]

In our recent work, we reported *in vitro* differentiation of P19 EC cells into IPCs by *C. intybus* leaf extract, which contains chicoric acid and chlorogenic acid.[2] Alfalfa has some phytochemical components, including coumarins, enzymes, alkaloids, flavonoids, organic acids, minerals, phenolic compounds, phytoestrogens, phytosterols, polyamines, amino acids, proteins, sterols, saponins, vitamins (e.g., vitamin K and C), and volatile.[64] Flavonoids, as phenolic compounds, have been shown to have anti-oxidant effects on many chronic diseases such as diabetes, cancer, Alzheimer's disease, and cardiovascular disease caused by oxidative stress.[65] The plant also contains copper, manganese, folate, and a high concentration of bioactive compounds, including saponins, coumarins, flavonoids, phytosterols, phytoestrogens, and alkaloids. Saponins exist in alfalfa have heart-protective effects due to their cholesterol-lowering activity.[66] Here, HPLC analysis of alfalfa extracts confirmed the presence of two critical flavonoids, chicoric acid and chlorogenic acid in FE and the presence of chlorogenic acid in LE (Supplementary materials, Table 2). It has previously indicated that chicoric acid is able to increase glucose uptake and insulin release by islets of rat pancreas and an insulin-secreting cell line, INS-1E.[67] In addition, the hypoglycaemic effect of chlorogenic acid has been reported in some *in vitro* and *in vivo* studies.[3,4,68,69] Some reports are showing that phenolic and flavonoid components of plants can promote proliferation and differentiation of stem cells through activating signaling pathways and inducing gene expression.[70–72] A natural flavonoid, apigenin, stimulated osteogenic differentiation of human MSCs by activating the JNK and p38 MAPK signal pathways.[70] Some reports confirmed that flavonoid quercetin induced osteogenic and pancreatic β-cell differentiation of MSCs.[72] The effect of flavonoid extract of a medicinal herb, *Cichorium intybus* L., to induce *in vitro* differentiation of P19 embryonal carcinoma (EC) stem cells into pancreatic β-cells has been reported in our recent study.[2] To the best of our knowledge, the present study is the first research on the *trans*-differentiation of mouse MSCs into pancreatic β-like cells by *M. sativa*." Therefore, natural compounds found in medicinal plants may have an essential role in treating chronic diseases, including diabetes. However, further researches are required to comprehend the precise effects of each component of the medicinal herbal extract on stem cells as a promising valuable source for cell therapy.

5. Conclusions

Overall, the results of the present study showed for the first time that *Medicago sativa* flower and leaf extract has the potential to induce *trans*-differentiation in MSCs into IPCs with some characteristics of pancreatic β-like cells. Therefore, in addition to ectodermal and mesodermal lineages, MSCs have the potential of *trans*-differentiate into endodermal lineages. Since β-cell mass and function are decreased in diabetes, cell replacement therapy using *in vitro* differentiated stem cells would be one of the best treatment approaches in regenerative medicine. Medicinal plants such as *M. sativa*, with their potential to influence stem cell proliferation and differentiation, may be helpful in treating diseases such as diabetes. However, further studies are needed to understand the underlying mechanisms that regulate IPCs differentiation of MSCs and support the rational use of *M. sativa* by humans.

Conflicts of interest

All the listed authors have read and approved the submitted manuscript. The authors declare that there is no conflict of interest. The material covered in this paper has not been reported previously and is not considered for publication elsewhere.

Funding

This work carried out in the Research Institute of Biotechnology was supported by a grant from Shahrekord University (grant number 140.248). The funders had no role in the design of the study, data collection and analysis, preparation of the manuscript, and decision to publish.

Authors' contributions

F.E. Conceived and designed the experiments; S.M., F.E., L.S., and G. Sh. Performed the experiments; F.E., and L.S. Analyzed the data; F.E., and L.S. Wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors would like to express profound thanks to Yasaman Esmaeili, MA in TEFL, for the critical edition of the manuscript. This study is reported in accordance with ARRIVE guidelines.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2022.02.002.

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