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ORIGINAL ARTICLE

Comparative assessment of proliferation and immunomodulatory potential of *Hypericum perforatum* plant and callus extracts on mesenchymal stem cells derived adipose tissue from multiple sclerosis patients

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Abstract

Background Mesenchymal stem cells-derived adipose tissue (AT-MSCs) are recognized for the treatment of inflammatory diseases including multiple sclerosis (MS). *Hypericum perforatum* (HP) is an anti-inflammatory pharmaceutical plant with bioactive compounds. Plant tissue culture is a technique to improve desired pharmacological potential. The aim of this study was to compare the anti-inflammatory and proliferative effects of callus with field-growing plant extracts of HP on AT-MSCs derived from MS patients.

Materials and methods AT-MSCs were isolated and characterized. HP callus was prepared and exposure to light spectrum (blue, red, blue-red, and control). Total phenols, flavonoids, and hypericin of HP callus and plant extracts were measured. The effects of HP extracts concentrations on proliferation were evaluated by MTT assay. Co-culture of AT-MSCs: PBMCs were challenged by HP plant and callus extracts, and Tregs percentage was assessed by flow cytometry.

Results Identification of MSCs was performed. Data showed that blue light could stimulate total phenols, flavonoids, and hypericin. MTT test demonstrated that plant extract in concentrations (0.03, 1.2, 2.5 and 10 µg/ml) and HP callus extract in 10 µg/ml significantly increased. Both HP extracts lead to an increase in Tregs percentage in all concentrations. In particular, a comparison between HP plant and callus extracts revealed that Tregs enhanced 3-fold more than control groups in the concentration of 10 µg/ml callus.

Conclusions High concentrations of HP extracts showed effectiveness on AT-MSCs proliferation and immunomodulatory properties with a certain consequence in callus extract. HP extracts may be considered as supplementary treatments for the patients who receiving MSCs transplantation.

Keywords Multiple sclerosis · Mesenchymal stem cell · St. John's wort · Callus extract

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Introduction

Multiple sclerosis (MS) is an autoimmune and inflammatory central nervous system (CNS) disorder in young people. MS is associated with loss of self-tolerance and dysregulation of the immune system. Despite numerous investigations, MS pathogenesis is unclear and no treatment has yet been identified (Yousefi et al. 2019b). Currently, most of the prescribed medications are immunosuppressants and can only slow down the progress of the disease. These immunosuppressants are also associated with severe side effects (Heidari et al. 2019). Mesenchymal stem cells derived adipose tissue (AT-MSCs) are shown to play anti-inflammatory roles (Yousefi et al. 2019c) and several studies revealed that AT-MSCs have immunomodulatory and neuroprotective properties. T regulatory cells (Tregs) are one of the main critical cells in self-tolerance and are involved in the immune regulation responses. Based on several studies, the number of Tregs decrease in MS patients which causes a functional defect in Tregs.

Therefore, therapies that lead to the enhancement of the Tregs population are being considered as candidates for the management and treatment of this autoimmune disorder (Fahimeh et al. 2013). Lately, a growing body of evidence introduces the potential of medicinal plants with anti-inflammatory properties in the treatment of MS (DEMİRCİ 2019). Traditionally, *Hypericum perforatum* (St John's wort) is one of the top-selling herbal plants being used for its anti-inflammatory, anti-depressant, antiviral, antioxidant, antimicrobial, hepatoprotective, and wound-healing properties (Silva et al. 2016). Moreover, several studies have considered HP as a useful plant in the treatment of neurological disorders such as mild-to-moderate depression, Alzheimer's, and Parkinson's diseases (Kraus et al. 2007). HP extract contains bioactive ingredients such as hypericin, phenols, flavonoids, and xanthones that are localized in the small black glandular structures placed in different parts of the plant (Gaid et al. 2016). These bioactive compounds are being affected by genetic, environmental conditions such as light, harvesting time, method of storage, and extraction. Nowadays, plant tissue culture provides an efficient and safe way to produce valuable herbal medication substances by the formation of callus (Gadzovska et al. 2013). A callus is a mass of plant parenchymal cells derived from differentiated plant tissue which has turned to undifferentiated mass cells and contains valuable herbal compounds (10). Studies have shown that culturing callus could improve ingredients by safe elicitors such as light intensity, medium salt, etc. (Tusevski et al. 2016). To date, the effects of the different elicitors on the accumulation of callus metabolites of HP in vitro culture have not been thoroughly investigated (Wu et al. 2016). Indeed, this is the first annotative report that investigates the development of HP culture under the influence of various spectral lights for the enhancement of biomass and callus. In this study, the effect of different doses of extracts obtained from HP callus and field-grown plants on the proliferation and immunomodulatory of AT-MSCs derived from MS patients was investigated.

Materials and methods

Plant materials

Seeds of HP were provided by the Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. Seeds were cultured in an experimental field and during flowering time, the aerial parts of the plants were harvested and were prepared for analysis.

Callus culture

Seeds of HP plants were initially surface sterilized using ethanol 70% for 30 s, and then with NaOCl 20% (v/v) for 15 min. Afterwards, they were rinsed with sterile deionized water for 3 times. Subsequently, sterile seeds were cultured in Murashigi-Skoog medium containing salts, B5 vitamins, 3% sucrose, 7% agar. The pH of the medium was adjusted to 5.7 before autoclaving (20 min at 120 ℃). Cultures were incubated in a growth chamber at 25 ± 1 °C, under 16 h' light (2500–3000 lx) photoperiod. The shoots that arose from seeds were used as an initial explant source for callus induction. Callogenesis was optimized using combinations of plant growth regulators after 28 days. Following callus induction, callus of almost identical size was exposed to various light regimes of blue, red, blue-red (1:1), and yellow (control) for 4 weeks to investigate the secondary metabolite production. Eventually, calluses were frozen in liquid nitrogen and stored at − 80 °C.

Extract preparation

Plant and callus extraction procedure

HP plant and callus extracts were dried in the oven for 24 h at 40 °C. To prepare extraction for analyzing total phenols and total flavonoids, powdered plants and callus were extracted separately by warm ethanol solvent (70%). The mixture was sonicated in an ultrasonic bath (30,000 Hz) for 30 min at ambient temperature. Supernatants were separated after centrifugation at 3000 rpm for 15 min and extracts were stored at 4 °C until analysis.

Total phenols content (TPC) assay

Phenolic content was measured by Folin–Ciocalteu's colorimetric method. Initially, 50 µl of ethanolic extract was added to 1700 µl sterile distilled water and was kept for 5 min and then the solution was mixed with 250 µl of 1:1 Folin–Ciocalteu's reagent. 500 µl aqueous Na_2CO_3 (20%) was added and the mixtures were determined at 725 nm using a UV absorption spectrophotometer (SP-3000 Plus). The total phenolic content was calculated using a standard calibration curve with a gallic acid equivalent (mg/ml stock solution. Sigma-Aldrich).

Total flavonoids content (TFC) assay

The content of flavonoids compounds in the dried sample was determined according to the modified calorimetrically method of AlCl3. Briefly, 100 µl ethanolic extract was diluted with 450 µl sterile distilled water. After 5 min, 60 µl aqueous $NaNO_2$ (5%), 60 of µl AlCl3 (10%), 400 µl NaOH (1n), and 450 µl distilled water was added to the mixture. The absorbance of mixtures was read at 510 nm and flavonoid content was expressed as mg Quercetin equivalent (mg/ml stock solution. Sigma-Aldrich).

Total hypericins determination

Total hypericin content was analyzed according to previously published method (Nosratabadi et al. 2016). Then, 100 mg sample powder mixed with chloroform for 15 min at room temperature. After removing the chloroform phase by vacuum, samples were extracted with the aqueous ethanolic solution (90%). The mixture was sonicated for 30 min in dark conditions at ambient temperature. The samples were centrifuged for 15 min (6000 rpm). The supernatant was placed in a tube to determine hypericin content. Hypericin absorption spectra were detected numerically using a spectrum fragment in a range of 400–700 nm. Strong absorbance of total hypericin was evaluated at 592 nm. Quantification was performed by using the hypericin standard (Sigma-Aldrich) calibration curve.

Isolation of AT-MSCs from MS patients and healthy individual

To obtain subcutaneous adipose tissue, five female MS patients and three healthy females were selected and informed consent was taken from all of them. The study was approved by the Ethics Committee and the Institutional Review Board of Mashhad University of Medical Sciences. The written informed consent was obtained from all subjects before conducting the experiments. The liposuctioned adipose tissue was immediately washed with an equal volume of phosphate-buffered saline (PBS, Gibco). Then, the suspended adipose tissue was digested with collagenase I (1 mg/ml; Worthington; USA) under gentle agitation for 45 min at 37 °C. Then Dulbecco's modified eagles (DMEM) medium supplemented with 10% FBS, 1% penicillin/streptomycin (100 units/ml)/100 µg/ml added into the suspension of collected cells, stopping the collagenase activity. The cell suspension solutions were cultured in T175 flasks (SPL Life Sciences; Korea) and were maintained in the incubator with humidity conditions with 5% CO_2 and 95% air at 37 °C. The medium was replaced to remove non-adherent cells every 2 days. When the monolayer cells reached about 90% confluence, were detached from the culture flask by applying 25% Trypsin–EDTA Gibco for more tests (Yousefi et al. 2020).

Characterization of AT-MSCs

AT-MSCs at passage three were characterized using CD markers according to the manufacturer's recommendations. AT-MSCs were cultured using 0.25% trypsin–EDTA and the trypsin activity was neutralized in a complete medium containing FBS. Further, 1×10^6 of AT-MSCs were washed two times with PBS and were suspended in PBS containing FBS 5%. According to the manufacturer protocol, AT-MSCs were incubated with antibodies (ebioscience, USA) in a dark place for 45 min at 4 °C. The negative antibodies were used for hematopoietic stem cell markers (CD45, D31, CD14, CD34, (HLA-DR) and positive for MSCs markers (CD90, CD73, and 105 CD). Also, isotype-control antibodies were used. Finally, AT-MSCs were acquired on a FACS Caliber flow cytometer (Becton Dickinson) and FlowJo software was used to analyze the data.

Differentiation capacity of AT-MSCs

In vitro adipogenic potential of AT-MSCs Adipogenic capacity was evaluated by induction media. Flask culture containing AT-MSCs with 80% confluences was trypsinized with trypsin/EDTA and the cells were centrifuged at 1200 rpm for 5 min. The cells were harvested in well plates with a density of 5×10^4 cells/well in presence of DMEM supplemented with 10% FBS. After AT-MSCs reached to 60% confluence, the medium was replaced with adipogenic differentiation media (AdipoDiff; StemMACS) including DMEM + 10% FBS, 2 mM L-glutamine, antibiotic (100 U/ml penicillin, 100 µg/ml streptomycin), 107 M dexamethasone, 50 g/ml of ascorbic acid and 10 g/ml of indomethacin. Plates were incubated in a humidified incubator (37 °C, 95% CO_2) for three weeks while the medium was refreshed every three days. Non-stimulated cells were cultured in only DMEM + FBS media as control. Finally, after 21 days, the plate contains differentiated adipose cells that were exposed

to Oil Red O staining and were microscopically assessed for appearing lipid vacuoles.

In vitro osteogenic potential of AT-MSCs To test the osteogenic ability, AT-MSCs were cultured in 6 well plates with the density of 5×10^4 cells/well and were incubated in a complete medium containing DMEM supplemented with 10% FBS. Next, AT-MSCs have attached the wells and obtained 90% confluence, the medium was replaced with osteogenic induction medium (OsteoDiff; StemMACS) containing 1 nM dexamethasone, 2 mM β-glycerophosphate, and 50 µM ascorbate-2-phosphate. For about 21 days, AT-MSCs were incubated at a humidified incubator (37 °C at 95% CO_2) while the induction medium was refreshed every three days. Following, transdifferentiated cells into osteogenesis, deposited calcium was stained by Alizarin Red and alkaline phosphatase according to recommended protocols.

In vitro chondrogenic potential of AT-MSCs To assay the chondrogenic potential of AT-MSCs, the pellet culture method was applied. Approximately 8×10^5 cells were washed twice and centrifuged at 1500 rpm for 5 min in a 15 ml centrifuge tube. After 24 AT-MSCs pellet cells were generated, the medium was changed with a chondrogenic induction medium containing 40 µg/ml proline (Thermo Fisher Scientific; USA), 10 ng of insulin, 10 ng/ml transforming growth factor-β3 (TGF-β3) (Peprotech; USA), 50 µg/ml ascorbic acid-2-phosphate, 100 nM dexamethasone and DMEM with 1% ITS + Premix (BD Biosciences; USA) and the induction medium was replaced every three days. Nearly after 21 days, transdifferentiated AT-MSCs to chondrocyte, the pellet cells were fixed with 4% paraformaldehyde. Afterward, the chondroitin cryo-sections were assessed by utilized toluidine blue and hematoxylin–eosin dye for the revealing of produced glycosaminoglycans (GAGs) and proteoglycans consequently of the prompted chondrogenic differentiation.

Cell proliferation assay by MTT

Cell proliferation of AT-MSCs was evaluated with the MTT test. AT-MSCs were cultured in 96 well flat-bottom plates (Iwaki SciTech; Japan) until reached 60% confluence. Subsequently, media of each well refreshed by complete medium (DMEM + 10% FBS) supplemented with HP callus and plant extracts included of different concentrations, in triplicate (0.002, 0.005, 0.01, 0.03, 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5 and 10 µg/ml and untreated AT-MSCs considered as a control). After 24 h, the medium was replaced with 100 µl MTT solution (0.5 mg/ml) and was incubated in a CO2 incubator for 3 h. Then supernatant of each well was removed and there was added 200 µl DMSO to each well forming black fumarate crystals. Absorbance was read by an ELISA reader at 570 nm.

Peripheral blood monocular cells (PBMCs) preparation

To provide PBMCs co-culture with AT-MSCs, blood samples of venous were collected from 5 healthy persons. Isolation of PBMCs was performed using Ficol (lymphoid, intotrain) to specify the density gradient by centrifugation. After the buffy coats layer was separated and rinsed with PBS containing 5% FBS. Trypan blue dye was used as a colorimetric method for microscopic evaluation of cell viability of PBMCs.

Treatment of co-culture of AT-MSCs and PBMCs with HP plant and callus extracts

AT-MSCs were harvested using Trypsin, washed twice with PBS and were cultured in DMEM containing 10% FBS to reach the final density of 1×10^4 cell/cm2. When the cells reached 60% confluency, became to investigate the synergic effects of AT-MSCs and HP plant and callus extracts on Tregs (TCD4+, TCD25+, FOXP3|+, CD127+). Afterward, PBMCs were co-cultured AT-MSCs (AT-MSCs: PBMCs at 1:5 ratios) and HP plant and callus extracts supplemented with different concentrations, in triplicate (2.5, 5, 10 µg/ml). Two untreated groups with AT-MSCs: PBMCs MS patient and AT-MSCs: PBMCs healthy were considered as control groups) added to each well separately. Cell culture plates were maintained in a humidified incubator at 37 °C with 95% CO_2 for 24 h. Flow cytometry assessment was done in triplicates for each subject (Table 1).

Statistical analysis

The data were reported as mean ± standard deviation (SD) and the comparison between values (i.e. analysis of variance [ANOVA]) using GraphPad Prism 8 software. The reported comparison is between the effect of different light spectrum on TPC, TFC, and total hypericin of HP plant and callus extracts. Then compared data between the treated AT-MSCs of MS patients and control groups, including the

Table 1 Study groups

Group	Treatment
Patient AT-MSCs: PBMCs	Control: untreated
Healthy AT-MSCs: PBMCs	Control: untreated
Patient AT-MSCs + PBMCs + HP-Plant extract	2.5 5 10 µg/ml
Patient AT-MSCs + PBMCs + HP-callus extract	2.5 5 10 µg/ml

untreated group with AT-MSCs: PBMCs patient and AT-MSCs: PBMCs healthy group. A p value of $p \leq 0.05$ was considered as the cutoff for significance.

Results

Callogenesis and effects of light spectrum on the enhancement of TPC, TFC, and total hypericin

In this study, callus induction was performed in Murashigi-Skoog medium using various combinations of plant growth regulators for 4 weeks (Fig. 1a, b). To survey the increase of bioactive compounds, the effects of the light spectrum including blue, red, mix of blue-red (50%:50%) and yellow as a control on callus culture was evaluated (Fig. 1c). Figure 2 shows a significant increase in cultures exposing to blue light spectrum in the accumulation of hypericin (2.39 mg/g dry weight) as compared with the experimental plant (1.49 mg/g dry weight) and callus control (0.91 mg/g dry weight) ($p \leq 0.05$). Also, the same result was obtained in the comparison between TPC of blue light (7.477 mg/g dry weight) and TPC of callus control (3.19 mg/g dry weight) ($p \leq 0.05$).

The results showed that the TFC of the plant (11.84 mg/g dry weight) and callus of blue light (16.64 mg/g dry weight) were significantly different compared to the TFC of callus control (10.66 mg/g dry weight) ($p \leq 0.05$). This graph indicates that blue light, as an efficient elicitor, stimulated the production of hypericin, TPC, and TFC in callus compared to other light quality (red, a mix of blue-red and control) and in comparison with these contents (TPC, TFC, and total hypericin) in the plant. Red spots as morphological properties where concentrating secondary metabolites have appeared in calluses under blue light (Fig. 1d). While the results of the current study red and mix of blue-red treats displayed less affection on the stimulating of hypericin, TPC and TFC compared to controls of callus and plant (Fig. 2).

Results of isolation and immunophenotyping of AT-MSCs

Adipose tissue samples were obtained from MS patients under liposuction surgery with an average volume of 200 ml/person. In all eight female subjects age- and gender-matched. The lipoaspirates from five female MS patients and 3 healthily female donors who had undergone liposuction surgery aged 26–35 (mean 33.7) years old, and weight 46–72 kg (mean ± SD = 58 ± 9.1). No bacterial, mycoplasma, and fungal contamination were found. Isolated AT-MSCs were cultured in T175 culture flasks for a week until 90% confluency. AT-MSCs grew in spindle shape and polygonal morphology was identified. Flow cytometry analysis of surface marker (Fig. 3), showed that AT-MSCs were positive for CD90, CD105, CD73, and negative for CD31, CD14, CD34, CD45, and HLA-DR.

Results of differentiation potential of AT-MSCs to adipocyte and osteocyte and chondrocyte

To further investigate AT-MSCs in the tri-lineage capacity, cells were cultured in the specific medium for three weeks. Eventually, AT-MSCs differentiation into adipocytes (Fig. 4a, b), osteocytes (Fig. 4c, d), and chondrocytes (Fig. 4e–h) were confirmed.

Doses of plant and callus extracts of HP stimulate the proliferative capacity of AT-MSCs

Generally, AT-MSCs were treated with variety of plant and callus extracts range of concentrations (0.002, 0.005, 0.01, 0.03, 0.07, 0.15, 0.3, 0.6, 1.2, 2.5, 5 and 10 µg/ml of HP plant and callus extracts and untreated AT-MSCs as a control). MTT test as a colorimetric assay was used to measure the proliferation of AT-MSCs. AT-MSCs proliferation cultured through enhancing concentrations of plant and callus extracts was increased. Indeed, the enhancement of proliferation was dose-dependent being induced by plant extract from (0.03–10 µg/ml) (Fig. 5),

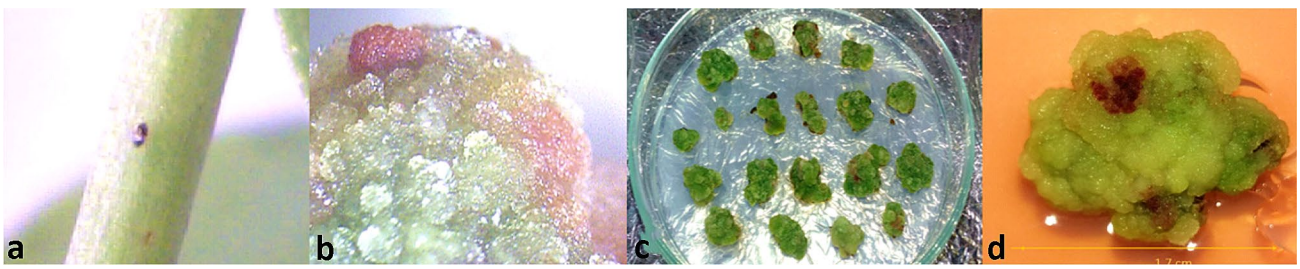

Fig. 1 Optical image of HP plant. **a** Black glandulars contain bioactive compounds which are located on the surface shoot (4X). **b** Callus were derived from the shoot (× 40). **c** Callus induction on MS (Murashigi-Skoog) medium. **d** Red spots have appeared on callus was treated by blue light

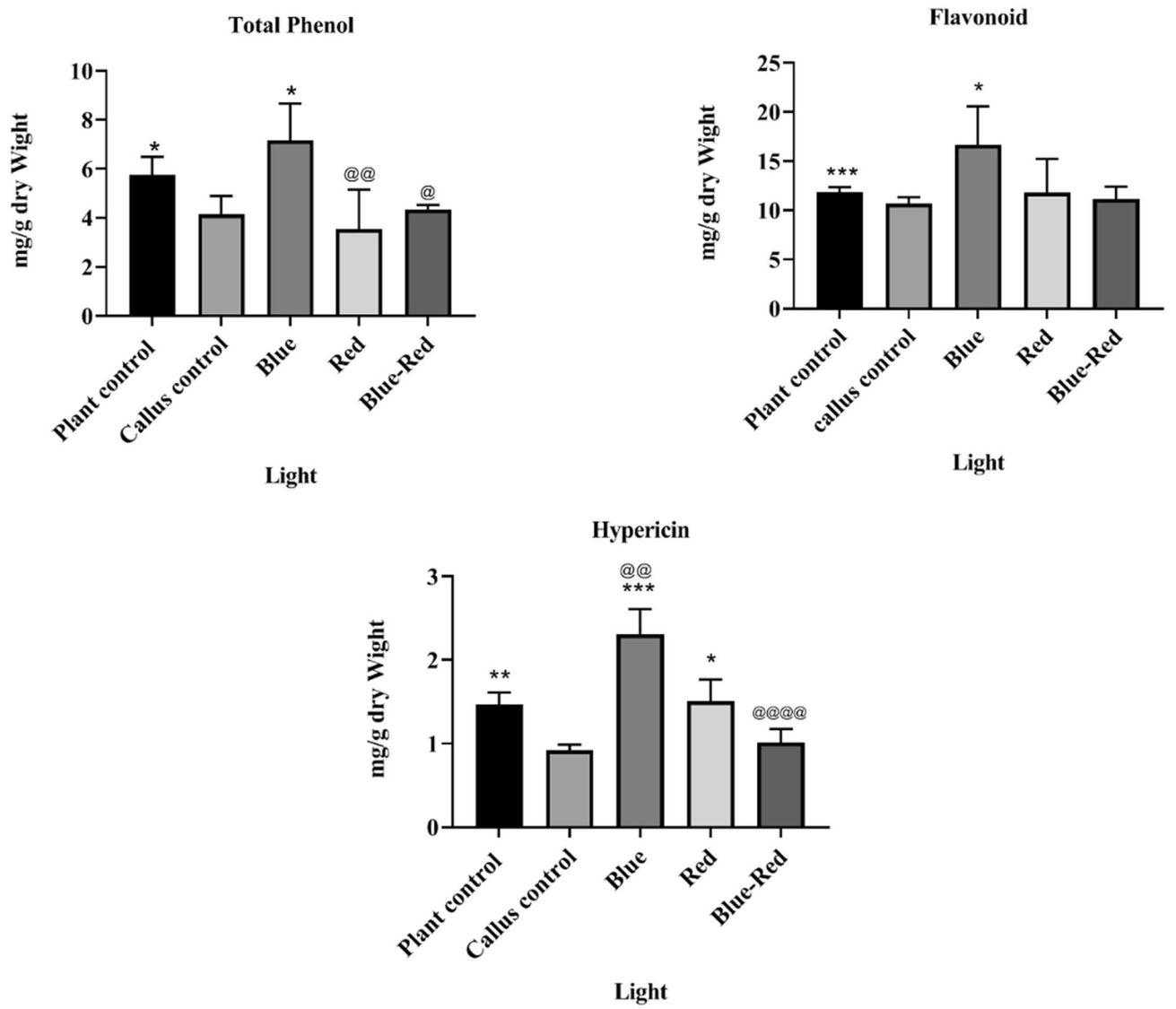

Fig. 2 Effects of light spectrum on TPC, TFC, and hypericin content. Blue light as an effective elicitor significantly stimulated hypericin to compare with callus control and plant. Also, TPC and TFC in blue light to compare with callus control showed a notable increase. *Significance vs. callus control ($p \leq 0.05$). @significance vs. plant control ($p \leq 0.05$). Data are expressed as mean ± SD for current experiments

and also in callus extract from lower doses (0.005–10 µg/ml) (Fig. 6). The results showed that higher concentrations of plant extract including (1.2, 2.5, and 10 µg/ml) and callus extract including (10 µg/ml) have significant effects on increasing optical density in comparison with the control group ($p \leq 0.05$). Furthermore, a comparison between low doses and untreated groups in then each treatment revealed that plant extract in a dose of (0.03 µg/ml) showed significantly increased ($p \leq 0.05$) and callus extract in lower dose (0.01 µg/ml) showed enhancing optical density however this change was not significant. Also, comparison between HP plant and callus extracts, showed higher optical density in a dose of 10 µg/ml significantly. The results were represented as the ratio of an optical density (OD) value (mean + SD) of the treated group to the OD of a control group.

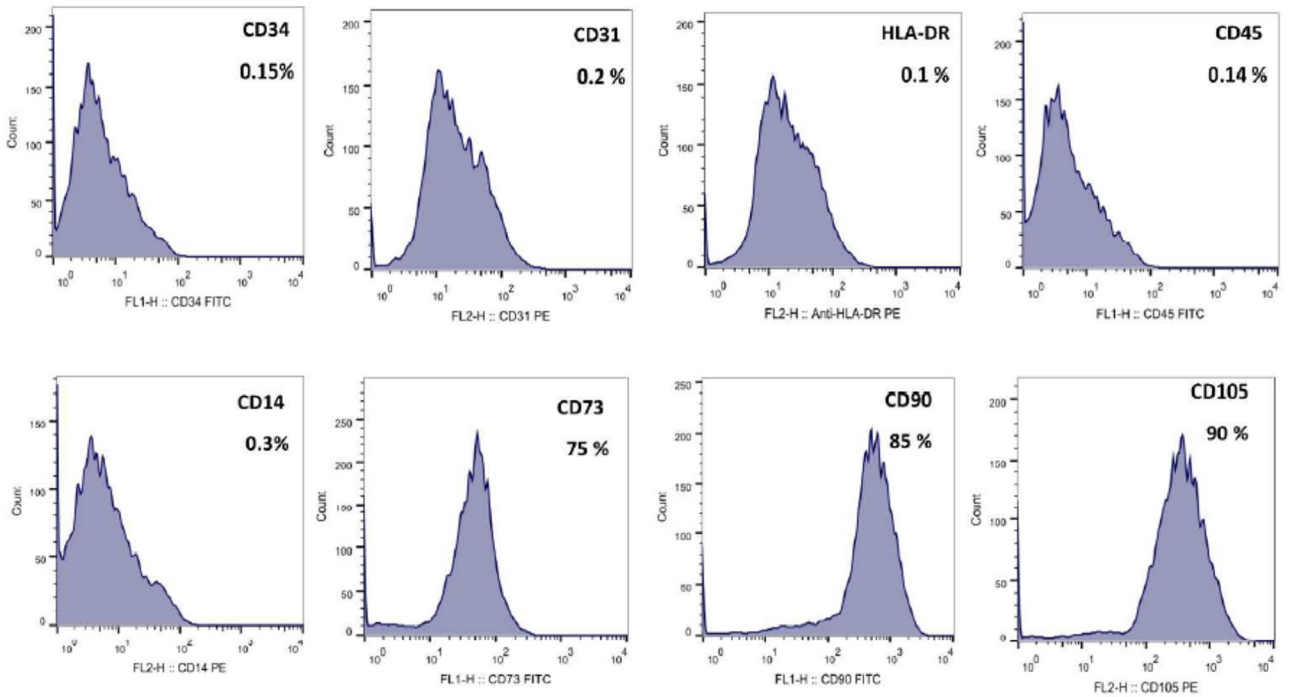

Fig. 3 Graphs were reported frequencies of surface markers to identify AT-MSCs. Flow cytometry experiment results from surface markers of AT-MSCs were positive for expression of CD90, CD73, CD105, and negative for CD31, CD45, CD34, CD14, and HLA-DR. Data properties occur at a rate of 1000 events/s.

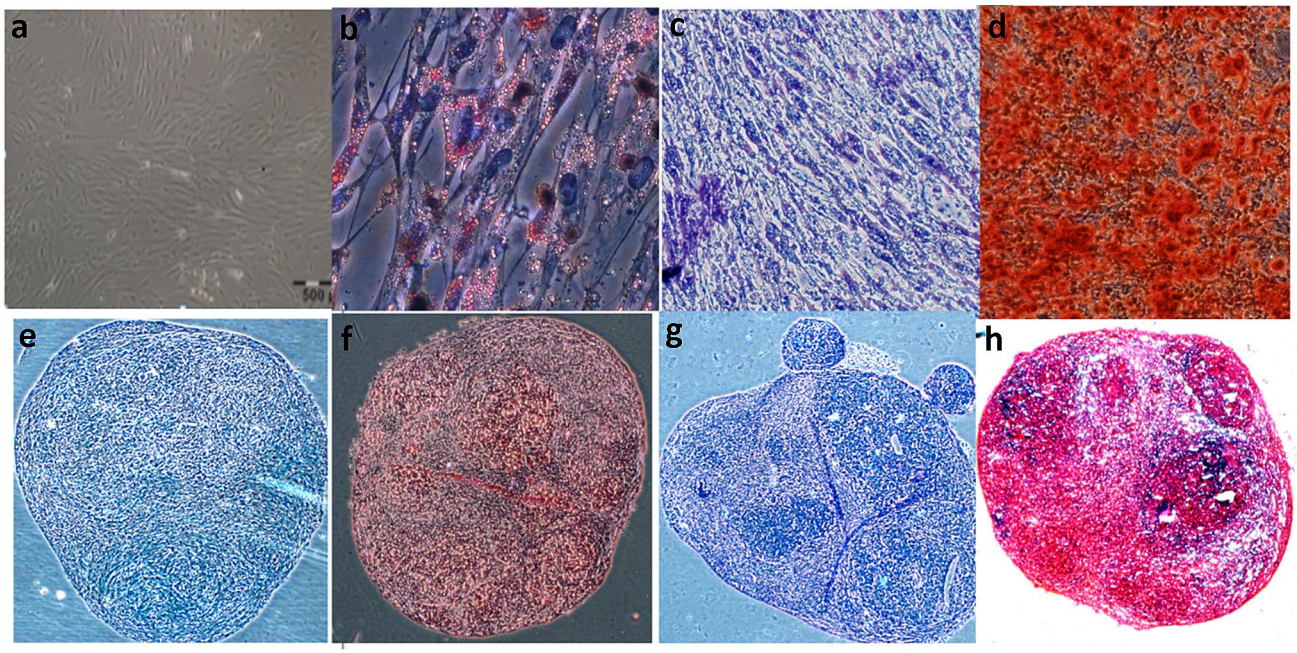

Fig. 4 Multi-differentiation capacity of AT-MSCs. Spindle morphology of AT-MSCs was investigated (**a**). The adipogenesis ability of AT-MSCs was investigated by appearing of lipid droplets after Oil Red O staining (**b**). Osteogenic differentiation of AT-MSCs was confirmed with emerging of mineralized matrix nodules after alkaline phosphatase (ALP) staining (**c**) or Alizarin Red staining (**d**). Chondrogenesis potential of AT-MSCs was evaluated by approving of glycosaminoglycan chains and proteoglycan accumulation after staining with Alcian Blue (**e**), Safranin (**f**), Toluidine Blue (**g**), and Hematoxylin Eosin (**h**). Scale bar **a**–**d** = 100 µM, and **e**, **h** = 500 µM

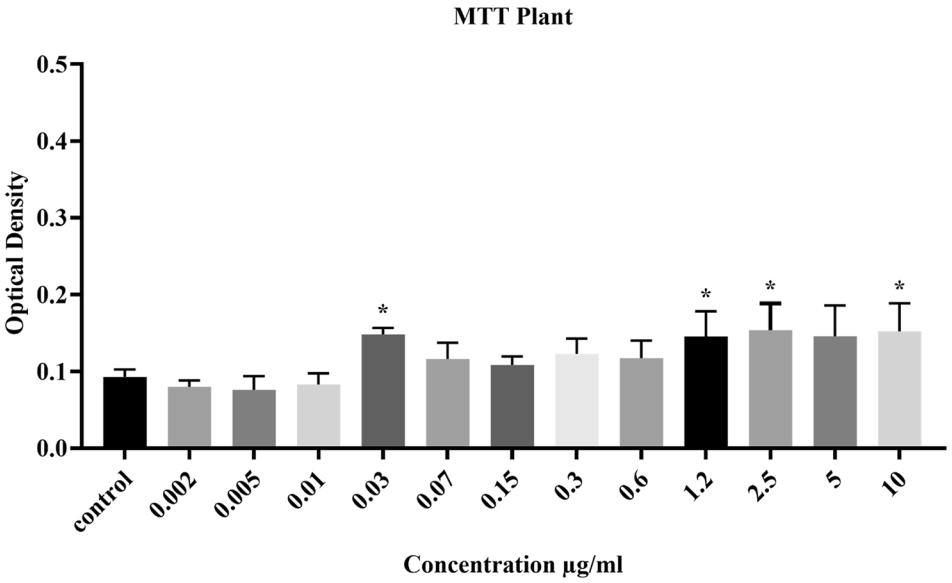

Fig. 5 Graph of AT-MSCs viability by MTT assay exposed to HP plant extract in range of concentrations (0.002, 0.005, 0.01, 0.03, 0.07, 0.15, 0.3, 0,6, 1.2, 2.5, 5 and 10 µg/ml, untreated as a control) measuring the optical density (OD) after 24 h. However, there is an increase in a dose-dependent manner of plant extract from 0.03 to 10 µg/ml. Results of HP plant extract with concentrations (0.03, 1.2, 2.5 and 10 µg/ml) showed significant increased to compare with control. Data were expressed as mean ± SD. *Significance vs. control ($p < 0.05$)

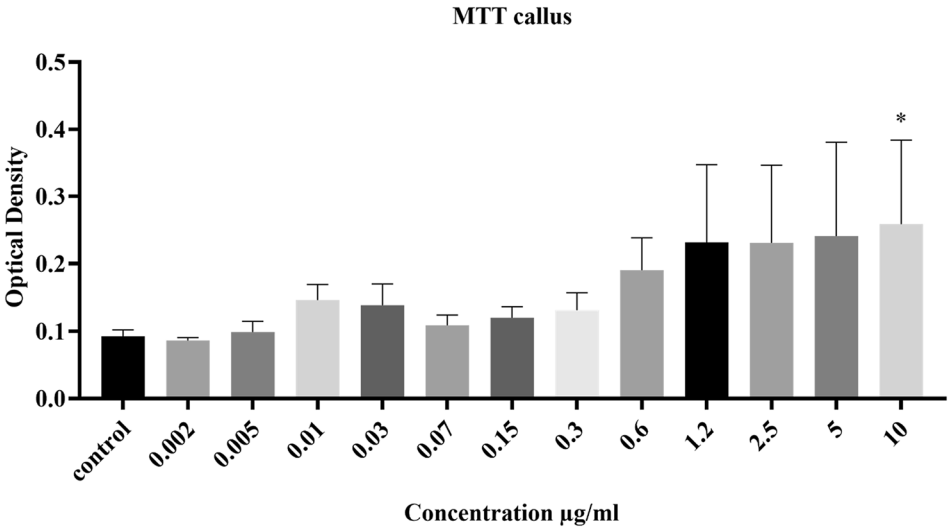

Fig. 6 Graph of AT-MSCs: MTT test exposed to HP callus extract in range of concentrations (0.002, 0.005, 0.01, 0.03, 0.07, 0.15, 0.3, 0,6, 1.2, 2.5, 5 and 10 µg/ml, untreated as a control) measuring the optical density (OD) after 24 h. Although data showed a dose-dependent manner that is increased from 0.01 to 10 µg/ml, the result of HP callus extract with a concentration of (10 µg/ml) displayed a significant difference to compare with control. Data were expressed as mean ± SD. *Significance vs. control ($p < 0.05$)

Effects of HP plant and callus extracts on Tregs (CD4 +, CD25+ FOXP3+ and CD127−) by flow cytometry

To compare between immunomodulatory effects of HP plant and callus extracts, AT-MSCs:PBMCs were co-cultured for 24 h in the presence of various concentrations of either HP plant and callus extracts (2.5, 5, and 10 µg/ml and two control groups). Then the percentage of Tregs (CD4+, CD25+, FOXP3+, and CD127−) were analyzed by the flow cytometry method. Data of dot plot graph in all concentrations of plant and callus HP extracts showed a rise in the percentage of Tregs populations compared to the two control groups (patient-AT-MSCs: PBMCs and healthy AT-MCs: PBMCs) (Fig. 7). The results of flow cytometry graphs (Figs. 7, 8) demonstrated that Tregs percentage significantly increased in HP plant extract of concentrations (2.5 and 10 µg/ml) in comparison with controls (patient AT MSCs and healthy AT: MSCs, respectively) ($p \leq 0.05$), however, the effectiveness of plant concentration at (2.5 µg/ml) were more significant ($p \leq 0.01$). Data of graph (Fig. 9) revealed that the effects of HP callus extract of concentrations (2.5 and 10 µg/ml) on Tregs population were remarkably enhanced in contrast with controls groups (patient AT: MSCs and healthy AT: MSCs, respectively) ($p \leq 0.05$) although callus extract at 10 µg/ml concentration was the most significant ($p \leq 0.0001$). In principle, data revealed that a dose with maximum effectiveness on Tregs was observed at 10 µg/ml concentration in plant and callus extracts. Also, a minimum increase of Tregs has appeared at the dose of 5 µg/ml. The results showed AT-MSCs: PBMCs showed synergic effect with callus extract on enhancing Tregs. Also, a comparison between the results of

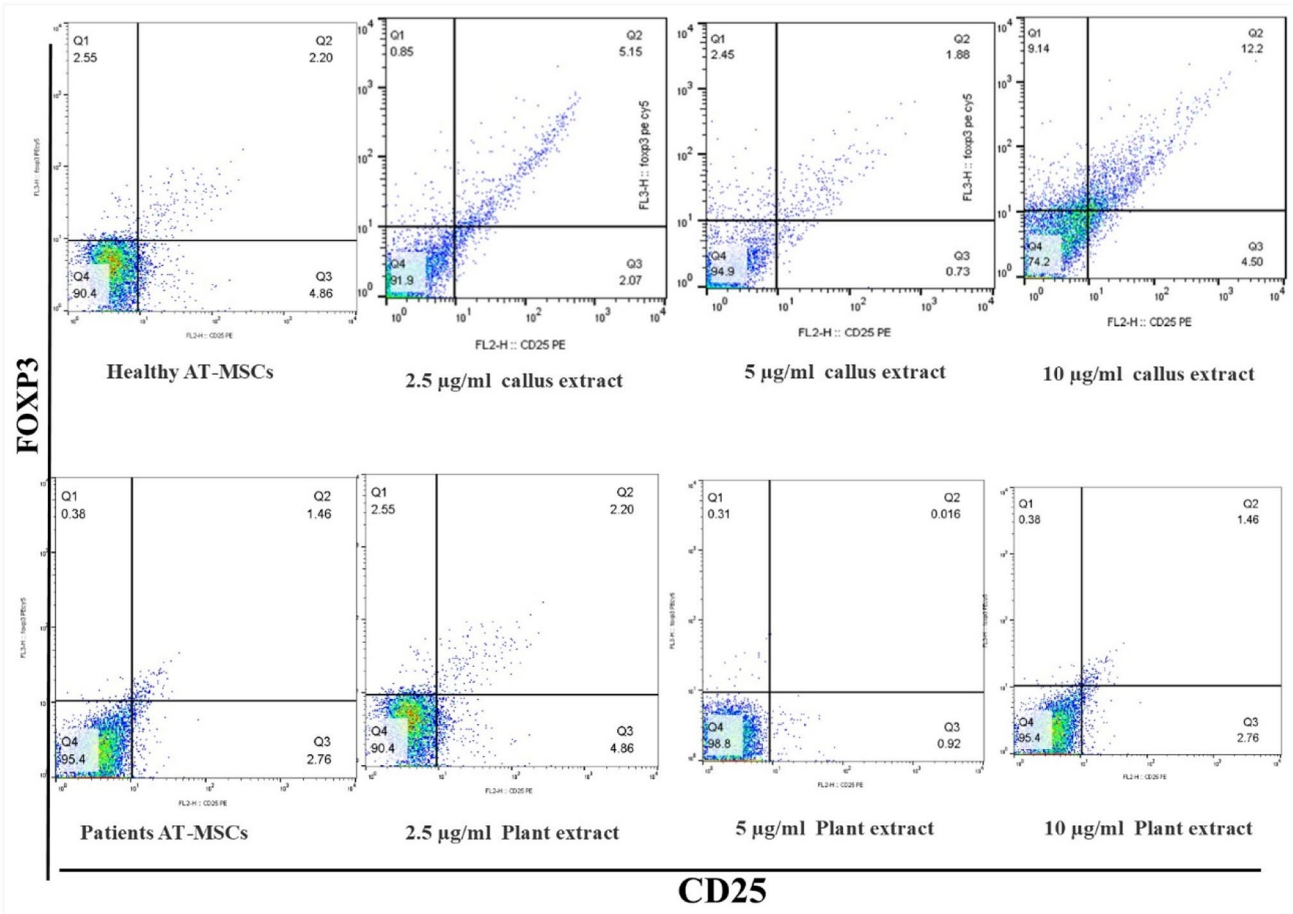

Fig. 7 Dot plot of flow cytometry analysis of co-cultured AT-MSCs: PBMCs after incubation with HP callus and plant extracts (2.5, 5, and 10 µg/ml) and two control groups. Staining was performed to detecting the percentage of Tregs expressed on the representative surface of CD marker (CD4+, CD25+, and CD127−) and intercellular marker (FOXP3 +). The result of HP callus extract with a concentration of (10 µg/ml) and plant extract with a concentration of (2.5 and 10 µg/ml) displayed significant differences to compare with control groups. Data were expressed as mean ± SD. *Significance with control groups ($p < 0.05$). **Significance with control groups ($p < 0.01$). ****Significance with control groups ($p < 0.0001$)

the plant and callus extracts expressed that Tregs enhanced in a high dose (10 µg/ml) of callus extract about three folds more than the control groups.

Discussion

Our previous study revealed that HP extract could inhibit the clinical course in the animal model of MS (Nosratabadi et al. 2016). In the current research, the primary goal was a further assessment of the comparative effects of different light spectrums on the enhancement of pharmacological ingredients (TPC, TFC, and total hypericin) of HP plant and callus extracts. The second goal was the comparison between proliferation and immunomodulatory properties of AT-MSCs which are isolated from the MS patients in vitro treated by HP plant and callus extracts. Data showed that between different light spectrums, blue light could produce more bioactive compounds (TPC, TFC, and total hypericin) in callus culture. Besides, our results revealed that HP plant and callus extracts increased cell proliferation ability of AT-MSCs and also expanded the number of Tregs in AT-MSCs: PBMCs mixture in all concentrations with a great outcome toward callus extract.

A growing body of evidence showed that HP extract is the well-known and top-selling medicinal plant of the current century. Also, HP extract has been used for the treatment of various diseases including mild to moderate depression, neurological disorders and inflammations Primary researches suggested most neuroprotective activity of HP extract (Tusevski et al. 2016) due to the presence of main bioactive pharmacological compounds especially flavonoid, phenol, and hypericin (Simic et al. 2015). Several studies have also been reported that HP extract possesses anticancer,

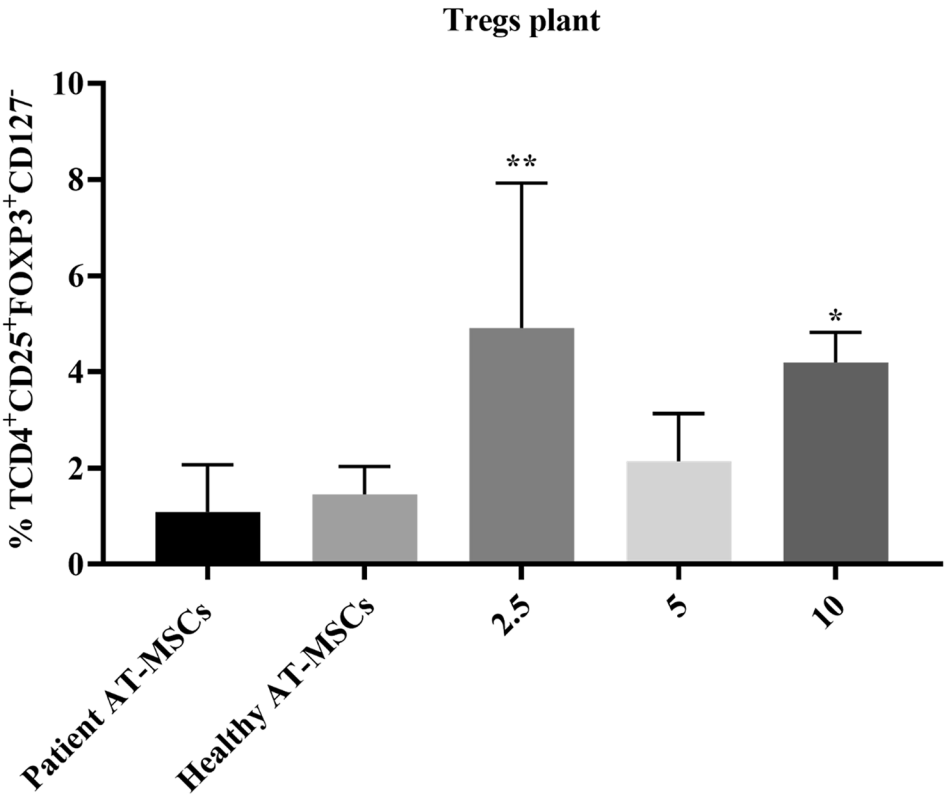

Fig. 8 Flow cytometry analysis of CD markers expression include extracellular (CD4+, CD25+, and CD127−) and intracellular (FOXP3+). Data of dot plot graph display AT-MSCs: PBMCs supplemented with HP plant extract concentrations (2.5, 5 and 10 µg/ml). The result shows that significantly affected HP plant extract concentrations of (2.5 and 10 µg/ml) on enhancing Tregs population compare to control groups (healthy AT-MSCs: PBMCs and patient AT-MSCs: PBMCs) ($p \leq 0.01$). *Significance with control groups ($p < 0.05$). **Significance with control groups ($p < 0.01$). Data were expressed as mean ± SD

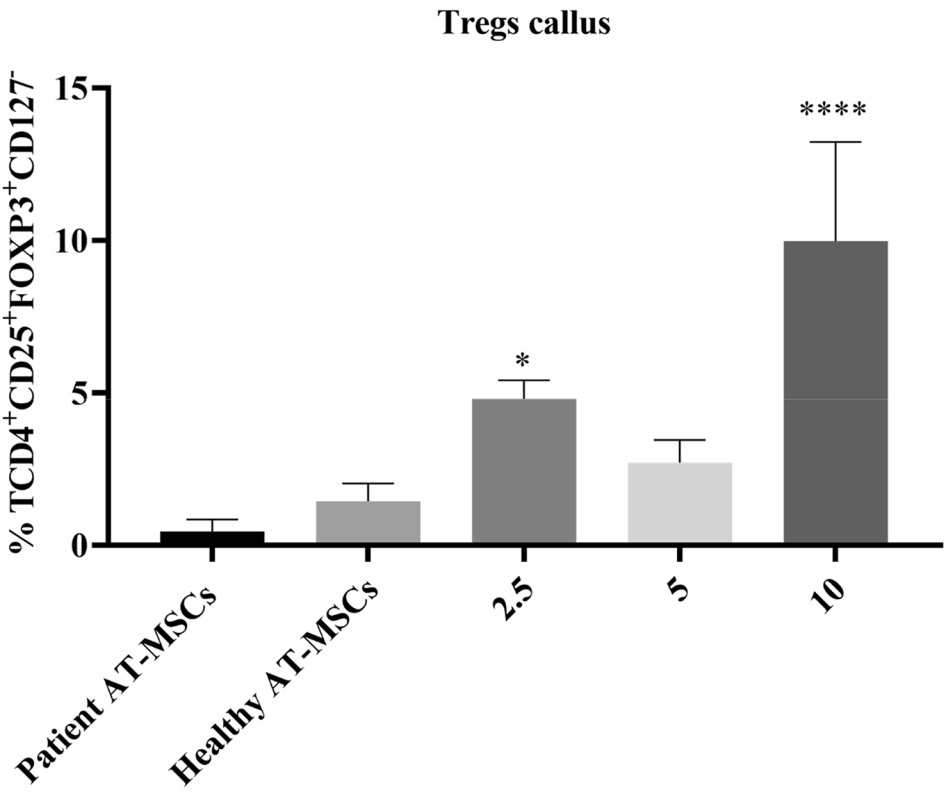

Fig. 9 Flow cytometry analysis of CD markers expression includes extracellular (CD4+, CD25+, and CD127−) and intracellular (FOXP3+). Data from dot plot graph showed AT-MSCs: PBMCs supplemented with HP callus extract concentrations (2.5, 5and 10 µg/ml). The result shows Tregs of HP callus extract concentrations (2.5 and 10 µg/ml) increased significantly compared to the control groups (healthy AT-MSCs and patient AT-MSCs: PBMCs). In contrast, Tregs population at 10 µg/ml increased about twofold more than two control groups. *Significance with control groups ($p < 0.05$). ****Significance with control groups ($p < 0.0001$). Data were expressed as mean ± SD

antioxidant and also known as immune-stimulating cell activations (Huang et al. 2014). However, the precise mechanism underlying HP plant pharmacology properties is still unclear. Furthermore, several studies demonstrated that HP extract compared to placebo or several drugs has upper or equal efficiency in the treatment of some diseases (Tocci et al. 2018). Likewise, depression extremely common in MS patients so use of HP extract maybe has therapeutic potential in MS disease treatment. There are growing studies about hypericin known as the major pharmacological component responsible for the anti-depressant and anti-inflammatory activities (Crespo-Bujosa and Gonzalez 2018). Inopportunely, the quality, and quantity of valuable pharmacological compounds and safety issues arose from field-cultivated plants is impressed by the variation in environmental condition, contaminations with heavy metals, microbes, and pesticides (Pradeep et al. 2020). A major bioactive compound in HP extract is hypericin which is localized in the small black glandular structures located on flower petals, stamens, leaves, and stems. Several studies showed that to increase and spread the manufacture of desired pharmacology products using in vitro elicitation cultures is a useful method (18). Indeed, plant tissue cultures serve as efficient, convenient and safe models for the mass production of valuable pharmacological metabolites (Gadzovska et al. 2013). Also this method is considered as an alternative method for plants with the slow growth rate and high price of herbal products (Achamlale et al. 2009). Plant tissue cultures not only avoid the disadvantages mentioned above but brought great benefits, shorten the growth cycle into weeks rather than years of growing plants in the field (Verpoorte et al. 2000). Because Callus was grown in sterile conditions without uptake of heavy ingredients, it is safe from toxicity affection. Callus has the potential for the production and accumulation of desired pharmaceutical compounds (Tocci et al. 2018). Previous studies found out that comparing HP field plant extract and callus extract exhibited differences in the quality and quantity of active compounds. For example, the study showed that eighteen xanthones (which are known to possess a wide spectrum of pharmacologic properties), were detected in the methanol extracts of HP callus cultures and none of them were identified in field ground plant extracts (Gadzovska et al. 2013).

Consequently, in our current study, the effects of the light spectrum (blue, red, blue-red, and yellow as control) were investigated on the accumulation of bioactive compounds (TPC, TFC, and total hypericin) of HP callus. Evaluating the comparison of these content between callus (under different light colors) and HP plant extract revealed that the effectiveness of blue light on the increase of TPC, TFC, and total hypericin. The defense system of the plant is activated against stress conditions which creating by biotic or abiotic elicitors. Some plants respond to elicitors as interaction with stress conditions by accumulating secondary or pharmaceutical metabolites with a variety of activities like antioxidant property (Yang et al. 2018). The pharmacologically active metabolites consist of phenols, flavonoids, terpenoids, etc. that production was stimulated with elicitors (Isah 2019). Incensement of potential for important phytochemical compounds, TPC, TFC, and also hypericin was performed by exposing the callus to different light quality as an abiotic elicitor. The current study showed that the blue light spectrum plays a key role in the enhancement of these valuable content (TPC, TFC, and hypericin) in the callus of HP. It seems that different four groups of the light spectrum (blue, red, blue-red, and yellow as control) as photoreactors performed photoconversion of protohypericin. Based on the previous studies and according to photoreactor protocols, hypericin as a photosensitizer pigment has more distribution in visible spectrum wavelengths. In addition, relatively blue light wavelength overlapping with a maximum band of hypericin absorption at λ592 can be considered as effective factors to increase hypericin. Due to the fixed life of plants as sessile nature cause to adapt their life to constraints. Fluctuating conditions make the plant acclimated using different mechanisms of regulation of gene expression. During evolution plants improved in several pathways of signal transduction in which responsible for responding to different ranges of a wavelength of light including far-red/red and blue light (Wu 2014). Molecular studies revealed that blue light plays an important role as an alternative promoter that causes to start of transcription start site (TSS) shift to alternative transcription start site. On the other hand, transcription is started from a distance transcription start site (dTSS) instead of TSS. This mechanism performs to avoid upstream open reading frame (uORF) for inhibiting translation. Because of this escaping, plants under blue light treatment have a higher level of translation efficiency and protein output from main open reading frame (mORF) than the plant grow in dark conditions (Gregory 2018). This process may be the widespread mechanism for regulating the response of the plant to different stress conditions and environmental factors. Recent reports showed that achieving a quantitative yield of hypericin, phenol, and flavonoid were improved by blue light spectrum (Gonçalves et al. 2017) and the influence of blue light in the related mechanism of increasing TPC and TFC were evaluated in root culture of HP (Sobhani Najafabadi et al. 2019). In agreement with our study, Ahmad et al. in 2016 reported that increasing TPC and TFC in blue light treatment agreed with our results in the callus culture of Stevia rebaudiana (Ahmad et al. 2016). Also, Sobhani Najafabadi et al. (2019) reported enhancement of TPC and hypericin in the first one week under blue light treatment. However, another study revealed that exposing root culture to red light after 5 weeks demonstrated more TFC than blue color. A similar result of increasing TPC and TFC under

blue color LED was documented on basil (*Ocimum basilicum*) and arugula (*Eruca sativa*) by Taulavuori (Taulavuori et al. 2018). In the comparison of total hypericin between plant and callus, Kirakosyan et al. (2008) showed an extract of callus had total hypericin at a higher level than the extract of a plant of the greenhouse. According to current experiments, depends on the desired compounds and duration of light treatment, blue light was investigated as an effective light quality enhancing pharmacologic compound, phenol and flavonoids, and especially hypericin.

Besides, MSCs therapy recently has great potential for regenerative therapies in MS disease (Llufriu et al. 2014). Adipose tissue is a unique assessable and rich source of MSCs gained using minimally invasive also has immunomodulatory effects on chronic inflammatory disease. The niche of MSCs is essential to improve the proliferation and immunomodulatory ability of MSCs (Kocan et al. 2017). Most notably, the application of MSC-based therapies would have more advantages and supports proliferation and cell viability activity (García-Sánchez et al. 2019). In this regard, suggesting that HP extract could have improved the proliferation of AT-MSCs but it should be considered a conflict of optimum dose could be more efficient (Mendi et al. 2018). Therefore, in this study, after culturing callus under blue light spectrum and obtained callus and plant HP extracts, the effects of it's on the proliferation of AT-MSCs were compared. Data showed all concentrations of both HP and callus could increase proliferation AT-MSCs; this increase was in a dose-dependent manner surprisingly, HP callus extract in lower doses could stimulate proliferation AT-MSCs comparison plant HP extract. Lu et al. (2004) reported an improved survival rate of neural cells effect of HP extracts on H2O2 trauma induced in a dose-dependent manner, at concentrations of 1–40 mg/mL in rat and oxidative stress model within 24 h treatment (Lu et al. 2004). Also Oliveira et al., 2016 investigated the neuroprotection or apoptosis effects of hypericin and quercetin as a major compound of HP on the transcription factor NF-kB, which is a key molecule involved in regulation and cellular processes, like neuronal survival and inflammatory response (Oliveira et al. 2016). At various concentrations of HP extract and hypericin of (1 and 10 μM) on cytotoxicity and expression of an apoptotic gene of hepatic cells dependent on the exposure time resulting in a pronounced decrease in viable cells relative to controls (Gadzovska et al. 2013).

Based on studies, the imbalance of T cell responses in the immune system plays an important role in the pathogenesis of the autoimmune disease (Fahimeh et al. 2013). In MS patient's autoreactive T cells proliferate expand can infiltrate into CNS and prompt inflammation and tissue impairment. Several studies showed that increased Tregs as a critical immunomodulatory agent could reduce autoreactive T cells leading to decreased inflammation and attack the CNS (Danikowski et al. 2017). Hence, methods that lead to increase Tregs as the immune regulator can make improvements in the immune regulation system (Yousefi et al. 2019a). In this study, the effects of HP plant extract and HP callus extract on the co-culture of PBMCs: AT-MSCs derived from MS patients were investigated. In our results, it was found that both HP plant and callus extracts in all doses rising in the percentage of Tregs populations. Astonishingly, after re-challenge with AT-MSCs: PBMCs which supplemented with HP plant and callus extracts, Tregs expansion in high doses shown better effectiveness and with a great outcome toward HP callus extract was threefold more than control. Similar to ours, in 2015 Nosratabadi et al. showed that HP extract could not only increase Tregs also reduced leukocyte infiltration in CNS of the EAE model (Nosratabadi et al. 2016). Furthermore, Novelli et al. (2016) revealed that HP extract and hyperforin as one of the bioactive compounds could prevent inflammatory cytokine signaling and apoptotic gene induction in a pancreatic cell line. Moreover, in other studies, HP extract in diabetes-induced learning and memory impairment in rats that were treated in doses (12 and 25 mg/kg) improved learning and memory and reversed learning and memory deficits (Hasanein and Shahidi 2011). A recent study in 2019 has proved that dexamethasone-induced diabetic depression in rats treated with HP extract at a dose of 100 and 200 mg/kg, body weight concurrent with dexamethasone injection possesses antioxidant, anti-inflammatory, and immunomodulatory effects (Elhadidy et al. 2019). Also, HP extract when used alone could reduce inflammatory and increased anti-inflammatory cytokines in dental palp-MSCs (Mendi et al. 2018).

According to our findings in this study, it seems that HP plant and callus extracts cultured in the blue light spectrum at high doses have more growth-promoting on AT-MSCs and anti-inflammatory effects.

Conclusions

All this considered, this research showed that AT-MSC co-cultured by PBMCs which were treated by HP plant and HP callus extract under blue light spectrum cultured could enhance the proliferation of AT-MSCs and have immunomodulatory effects by expanded Tregs percentage. The results of this research have several important implications for more research. Further in vivo and controlled trial studies needs to clarify the exact mechanisms of HP and MSCs interaction in MS patients and other neurologic diseases.

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Declarations

Conflict of interest The authors have declared that there is no conflict of interest to report. This research was financially supported by a Grant (921082) from the research council of Mashhad University of Medical Sciences (MUMS).

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