World Cancer Research Journal WCRJ 2020; 7: e1508

TRIFOLIUM PRATENSE EXTRACT INDUCES APOPTOSIS AND DECREASES NITRIC OXIDE SECRETION IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELL

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Abstract – *Objective:* *Angiogenesis plays a critical role in tumor growth and metastasis. Endothelial cell proliferation has been used as an* in vitro *model of angiogenesis.* Trifolium pratense *was suggested for cancer treatment in traditional medicine and scientific report. The present study was designed to investigate the effect of* T. pratense *hydroalcoholic extract on the human umbilical vein endothelial cell (HUVEC) apoptosis and nitric oxide (NO) secretion* in vitro.

Materials and Methods: *Hydroalcoholic extract of* T. pratense *was prepared and its different concentrations (0, 12.5, 25, 50, 100, 200, 400, 800 µg/ml) were used for cell treatment. Cell viability was assessed by trypan blue, MTT and lactate dehydrogenase methods. Furthermore, nitric oxide (NO) secretion of the cells was measured by Griess reaction, and their apoptosis was evaluated by fluorescent dyes staining. One-way ANOVA was used for data analysis.*

Results: *After 24, 48 and 72 hours treatments, the IC50 values were 1069.35, 174.70 and 68.07 µg/ml, respectively.* T. pratense *extracts exerted a significant difference between the groups treated compared to the control group (*p*<0.05) and increasing the dose significantly decreased cell viability.* T. pratense *extract significantly increased LDH in dose- and time-dependent manners in the cell culture medium (*p*<0.05). NO decreased in a dose-dependent manner, and cell apoptosis was increased significantly (*p*<0.05).*

Conclusions: T. pratense *exerts a cytotoxic effect on HUVEC in dose- and time-dependent manner. It can be considered as an anti-angiogenesis agent and potentially beneficial for further development of new anti-cancer agents.*

KEYWORDS: Trifolium pratense, *Apoptosis, Nitric oxide, HUVEC, Angiogenesis.*

INTRODUCTION

Angiogenesis is a physiopathological process which includes the development of new capillaries from pre-existing vessels and has pivotal roles in growth, development, and wound healing. Endothelial cells line the inner surface of blood vessels and play a fundamental role in formation of new vessels during vasculogenesis and angiogenesis[1]. Angiogenesis is also required for tumor growth and metastasis, and it is an important factor in cancer progression. It is a vital step in the transition of tumors from a benign state to a malignant form, so inhibition of angiogenesis can be useful in the cancer treatment[2,3].

Nowadays, the use of anti-angiogenesis agents is a highly promising therapeutic approach for cancer. Angiogenesis inhibitors consist of direct inhibition of vascular endothelial cells and indirect inhibition of angiogenesis inducers. More than forty anti-angiogenic agents are being tested in human cancer patients in

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clinical trials[3]. In this regard, many chemical and natural compounds have been reported to be able to inhibit endothelial cell proliferation, and several *in vivo* and *in vitro* studies have been investigating the effect of natural compounds derived from food sources, plants and marine organisms on angiogenesis[1,4,5].

Trifolium pratense L., a member of Leguminosae family, is a short-lived biennial plant, which has been used as a valuable food for humans. It is probably native to Europe, Western Asia, and northwest Africa, but it has been naturalized in other continents[6]. Many isoflavones extracted from *T. pratense* are available nowadays as dietary supplements [7]. This plant has also been suggested in the traditional medicine as a treatment for some human diseases such as whooping cough, asthma, eczema and certain eye diseases[8,9].

A study documented the chemical profile of *T. pratense* extract using the high-performance liquid chromatography–ultraviolet (HPLC-UV) chromatogram. The results showed that *T. pratense* extract was composed of isoflavones, flavonoids, pterocarpans, coumarins and tyramine[8]. Its main isoflavones are formononetin, genistein, biochanin A, daizdein, calycosin, pratensein, prunetin, pseudobaptigenin, afrormosin, texasin[10].

Despite current remarkable progress in cancer therapy, it remains the major cause of mortality worldwide. So, the discovery and development of new strategies for cancer treatment is very urgent. *T. pratense* has been suggested for cancer treatment in traditional medicine. The inhibitory effect of *T. pratense* extract on chorioallantoic membrane assay of fertilized hen's eggs was reported[11]. Our previous works showed that *T. pratense* hydro-alcoholic extract inhibited the *in vitro* proliferation of breast cancer cell lines (MCF-7 and MDA-321), glioblastoma multiform (U87MG) and prostate cancer (LNCap and PC3)[12-14]. The present study was designed to evaluate the *in vitro* effect of *T. pratense* extract on HUVEC viability, apoptosis and NO production.

MATERIALS AND METHODS

Human umbilical vein endothelial cell (HUVEC) was obtained from the National Cell Bank of Iran (NCBI). Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and trypsin were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified eagle medium/Ham's F12 (DMEM/F12) were purchased from Gibco (Gibco BRL, Rockville, MD, USA). All experiments were repeated independently at least three times and were performed in triplicates.

HUVEC line was grown in cell culture flasks containing DMEM/F12 supplemented with 10% FBS without antibiotics. Cells were maintained at 37°C in a humidified chamber containing 5% CO_2. The cell line was treated with *T. pratense* extract (12.5, 25, 50, 100, 200, 400 and 800 µg/mL). *T. pratense* seeds were cultured in spring in a farm and identified in terms of species by a botanist (School of Pharmacy, Kermanshah, Iran). Herb extract was prepared using percolation method as described in previous work[15].

Cell viability assay

HUVEC cells were cultured in 24-well plates at 7×10^4 cells/well and incubated overnight. Then, the cell culture medium was replaced with fresh serum-free medium containing various concentrations of *T. pratense* extract. The cells were incubated for 24, 48 and 72 hours. Subsequently, the cells were harvested by trypsinization and were resuspended in PBS. Then the cell suspension was mixed with an equal volume of 0.4% trypan blue. The number of live cells (unstained) over the total number of cells was calculated as the percentage of viability.

HUVECs were cultured in 96-well plates at a density of 1.5×10^4 cells/well. Then media containing different concentrations of the extract were added to separate wells. After 24, 48 and 72 hours of treatment, the media were removed and 30 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 additional hours. 100 µL of DMSO was added to dissolve the formazan crystals produced by living cells at room temperature for 10 minutes. The optical density (OD) of final solutions was measured using an ELISA reader at 570 nm with a reference wavelength of 630 nm. The percentage of cell viability was calculated according to the following formula[16]:

Cell viability (%) = [OD570, 630 (sample)/ OD570, 630 (control)] ×100

The half maximal inhibitory concentration IC_{50} values of *T. pratense* extract were calculated by nonlinear regression using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA).

Lactate dehydrogenase (LDH) assay

HUVECs were cultured in 24-wells plate and incubated overnight. Culture media (500 µl) containing different concentrations of *T. pratense* extract were added to each well, and the plates were incubated for 24, 48 and 72 hours. Then, 100 µl of medium from each sample was transferred to other plates and LDH activity was measured using Cytotoxicity Detection Kit (Roche Chemical Co., Mannheim, Germany) according to the manufacturer's procedures. Finally, the OD at 490 nm with a reference wavelength of 690 nm for each sample was measured[17].

Nitric oxide (NO) assay

Griess reaction was used for evaluation of the effect of *T. pratense* extract on NO production by HUVEC. After treatment with the different concentrations of the extract for 48 hours, the culture medium from each sample was collected and NO concentration was measured as described in previous work[18].

TUNEL staining

Apoptosis was evaluated by labeling the 3′- hydroxyl termini in DNA fragments using an *In-Situ* Cell Death Detection Kit, AP (Roche Diagnostic; Mannheim, Germany) as described in the manufacturer's instructions.

AO/EB double staining

For observation of the intact, apoptotic and necrotic cells, AO/EB double staining was performed. AO passes through the plasma membrane of cells and emits a green fluorescent light. EB only passes from the plasma membrane of cells when cytoplasmic membrane integrity is lost and emits a red fluorescent light. EB emission dominates over AO. Therefore, live cells show uniform green nuclei and early apoptotic cells have yellow nuclei with fragmented chromatin. Late apoptotic cells have fragmented chromatin and orange nuclei, and necrotic cells have solid orange nuclei. HUVEC was cultured in 24-well plates and treated with *T. pratense* extract. After 48 hours, cells were stained with mixture of AO/EB dye containing 100 µg/ml of AO and 100 µg/ml of EB in PBS and observed under a fluorescent microscope[19].

Real-time PCR

Total RNA from HUVEC, treated with *T. pratense* extract for 48 hours, was prepared by total RNA isolation kit (DENAzist, Tehran, Iran) and the quantity and quality of the extracted RNA were tested by nano drop and gel electrophoresis. The complementary DNA (cDNA) synthesis was carried out using cDNA synthesis kit (Viventis Technologies, Selangor DE. Malaysia). Real-time PCR was performed using SYBR Premix Ex Taq technology (TaKaRa Bio Inc., Otsu, Shiga, Japan).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control and the fold change in relative expression of each target mRNA was calculated on the basis of comparative ct by 2 to the negative power of ΔΔct. Thermal cycler conditions were 15 min at 50°C for cDNA synthesis, 10 min at 95°C followed by 40 cycles of 15 s at 95°C to denature the DNA, and 60 s at 60°C to anneal and extend the template. The primer sequences were as follows:

Bax forward:
5′-CCTGTGCACCAAGGTGCCGGAACT- 3′
and
reverse:
5′- CCACCCTGGTCTTGGATCCAGCCC- 3′,

Bcl-2 forward:
5′- TTGTGGCCTTCTTTGAGTTCGGTG- 3′
and
reverse:
5′- GGTGCCGGTTCAGGTACTCAGTCA- 3′,
P53: forward:
5′- TAACAGTTCCTGCATGGGCGGC- 3′
and
reverse:
5′- AGGACAGGCACAAACACGCACC- 3′
and
caspase 3 forward:
5′- CAAACTTTTTCAGAGGGGATCG- 3′
and
reverse:
5′- GCATACTGTTTCAGCATGGCAC- 3′.
GAPDH Forward:
5 -TCCCTGAGCTGAACGGGAAG- 3
Reverse:
5 – GGAGGAGTGGGTGTCGCTGT - 3

Statistical analysis

All data are presented as mean ± standard deviation (SD) of three independent experiments. Statistical evaluation was done using one-way analysis of variance (ANOVA) with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software and *p*-values of less than 0.05 were regarded as statistically significant.

RESULTS

Cell viability

The results of trypan blue staining and MTT assay at 24, 48, and 72 hours showed a significant difference among the groups treated with *T. pratense* extract (12.5, 25, 50, 100, 200, 400 and 800 µg/ml) compared to the control group. Increasing the dose significantly decreased cell viability (Figure 1A, B) ($p<0.05$). *T. pratense* extract reduced HUVEC viability in dose- and time-dependent manner. The IC_{50} values at 24, 48- and 72-hours treatments were 1069.35, 174.70 and 68.07 µg/ml, respectively. Measurement of LDH activ-

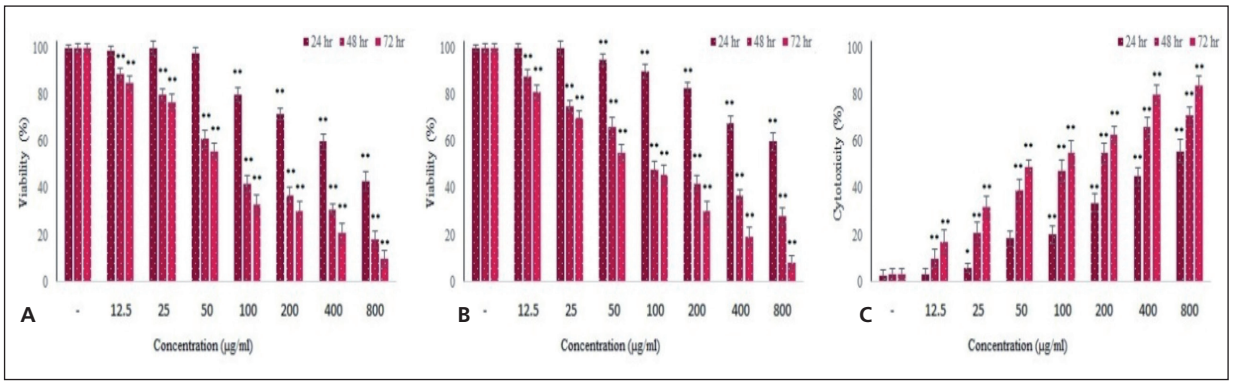

Fig. 1. The effect of Trifolium pretense on HUVECs. Cells were treated with indicated concentrations of extract for 24, 48, 72 hr. Cells viability was determined using the *(A)* trypan blue staining *(B)* MTT assay and *(C)* LDH release measurement as described in the Methods section. Control wells were treated with the equivalent amount of a medium alone. The data are expressed in terms of percent of control cells as the means ± SD. (**p*<0.05; ***p*<0.01 compared with control).

ity in a cell culture medium revealed that *T. pratense* extract significantly increased LDH release in dose- and time-dependent manners (Figure 1C) ($p<0.05$). Therefore, cell death mediated by *T. pratense* is accompanied by plasma membrane damage.

NO level

The effects of different concentrations of *T. pratense* extract on HUVEC after 48 hours of treatment showed a dose-dependent decrease in NO production. The difference compared to the control group was significant for all concentration except 12.5 µg/ml ($p<0.001$) (Figure 2).

Apoptosis assay

The apoptosis index of HUVECs treated with different concentrations of *T. pratense* extracts at 48 hours showed that *T. pratense* increased apoptosis significantly in a dose-dependent manner ($p<0.05$). Apoptotic cell death was quantified and presented as a percentage (Figure 3). Furthermore, morphological changes in apoptotic cells, including cell shrinkage, chromatin condensation and nuclear fragmentation were detected using fluorescent dyes. As presented in Figure 4, live cells with normal morphology were abundant in the control group, whereas early apoptotic cells were seen in the 12.5 µg/ml dose. Both early and late apoptotic cells were observed in cultures treated with 25, 50 and 100 µg/ml, and in the 200, 400 and 800 µg/ml doses most of the cells were in the late apoptotic stage. Therefore, HUVEC apoptosis increased with *T. pratense* extracts in a dose-dependent manner.

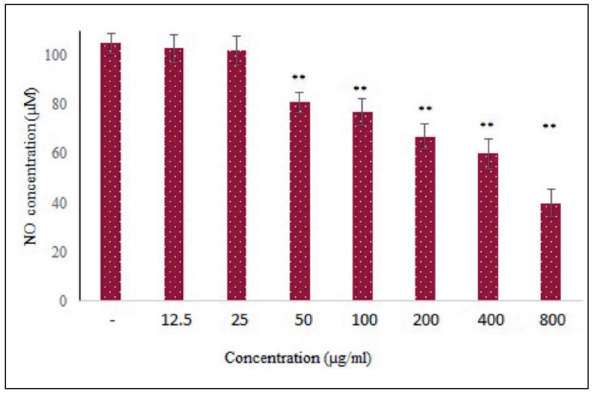

Fig. 2. The effect of Trifolium pretense on NO production by HUVECs was measured by Griess reaction. Control wells were treated with the equivalent amount of a medium alone. The data are expressed in terms of percent of control cells as the means ± SD. (**p*<0.05; ***p*<0.01 compared with control).

Real-time PCR

Expressions of some apoptotic genes were evaluated using Real-time PCR. As shown in Figure 5, P53 was up-regulated in cells treated with *T. pratense* extract. The results also suggested a down-regulation of BCL-2 and up-regulation of BAX mRNA expression after exposure to *T. pratense* extracts (Figure 5B and C). Exposure of cells to *T. pratense* extracts led to increased mRNA expression of Caspase 3 gene (Figure 5D).

DISCUSSION

The study indicated that HUVEC viability was decreased significantly in a time- and dose-dependent manner by different concentrations of *T. pratense* hydro-alcoholic extract (12.5, 25, 50, 100, 200, 400 and 800 µg/ml). Furthermore, their NO secretion was decreased, and the cell apoptotic index increased significantly. Morphological changes and gene expression analysis (Real Time PCR) were in consistent with other data.

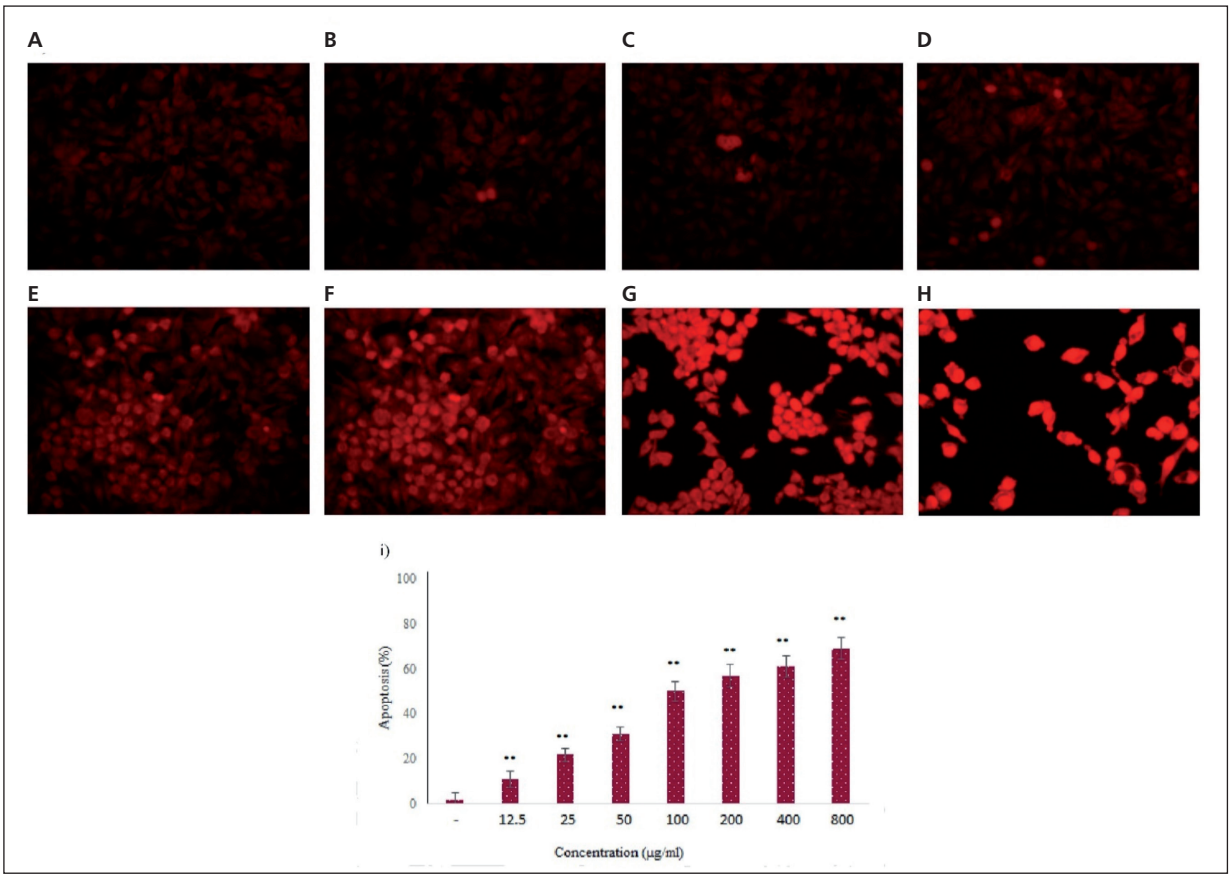

Fig. 3. The effect of Trifolium pretense on apoptotic potential in HUVECs was evaluated using TUNEL staining. *A*, control group; *B*, in the presence of 12.5; *C*, 25; *D*, 50; *E*, 100; *F*, 200; *G*, 400; *H*, 800 µg/ml of T. pratense extract for 48 hr; and *I*, columns mean percentage of apoptotic cells from three independent experiments performed in triplicate. Control cells treated with medium without FBS. *p*-values were determined using one-way-ANOVA (**p*<0.05 compared with control; ***p*<0.01 compared with control).

Angiogenesis is an important factor in the progression of cancer. The importance of endothelial cell migration and proliferation has been shown in several studies. The induction of endothelial cell apoptosis is an important anti-angiogenic mechanism[20] and can be used to inhibit new blood vessel formation in tumors[21]. Studies indicated that some natural components founding in the *T. pratense* such as flavonoids, coumarins and pterocarpans have generally shown anti-proliferative effects. The results of our study showed that the *T. pratense* extract increased apoptosis of HUVEC. Moreover, other works as the present study showed that anti-cancer effect of flavonoids is mediated by activation of apoptosis.

From the pterocarpans family, indigocarpan has shown anti-proliferative activity in human cancer cell lines via induction of caspase-dependent apoptosis pathways. Furthermore, NO exerts many important functions in the angiogenesis. It is a pro-angiogenic agent and increases endothelial cells' proliferation and enhances their migration[22-24]. Angiogenesis progress needs the synthesis of endothelial NO[25,26]. Data showed that *T. pratense* extract decreased NO secretion.

An *in vivo* study with a chick chorioallantoic membrane (CAM) showed that some isolated isoflavones from soybeans, including genistein, daidzein and orobol, inhibit angiogenesis, albeit with different potencies. They also inhibited human endothelial cell proliferation[27]. Among these isoflavone compounds, genistein was the most potent inhibitor of angiogenesis *in vitro* and *in vivo*[28]. Flavonoids, the most abundant polyphenols in our diet, have been proposed to act as a chemo-preventive agent in numerous epidemiological studies and have been shown to inhibit angiogenesis and proliferation of tumor cells, and endothelial cells *in vitro*[29]. An *in vivo* study showed the anti-angiogenic activities of propolis extract, and the major components found in the extract from the red propolis were characterized as isoflavonoids and pterocarpans[30]. According to these data, some effects of *T. pratense* can be attributed to its isoflavones, flavonoids, and pterocarpans.

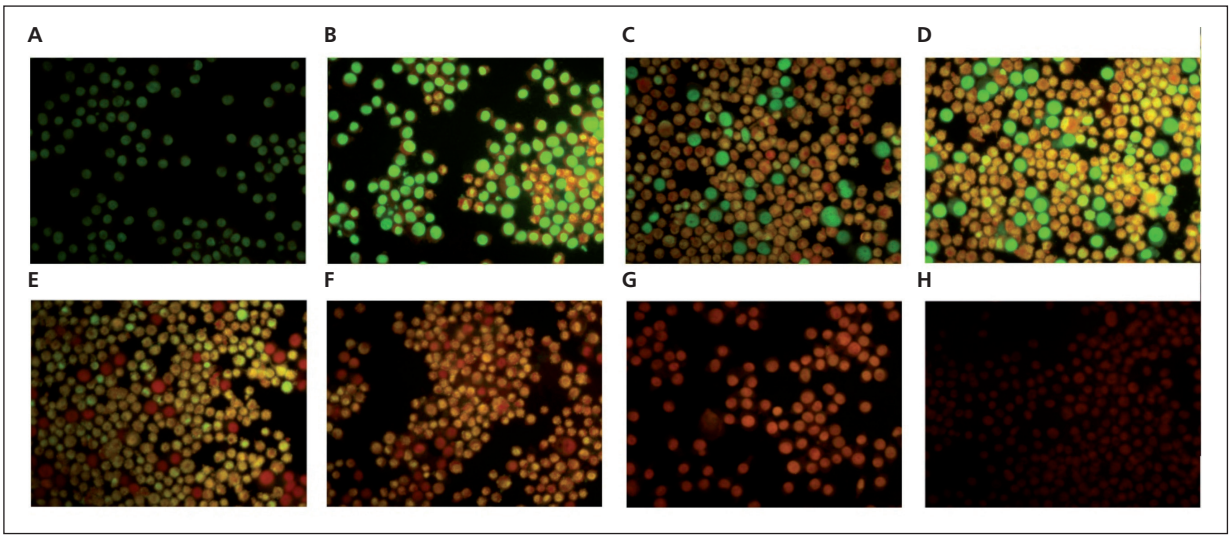

Fig. 4. HUVEC cells were stained by AO/EB and observed under fluorescence microscope: *A*, control group; *B*, in the presence of 12.5; *C*, 25; *D*, 50; *E*, 100; *F*, 200; *G*, 400; and *H*, 800 µg/ml of Trifolium pretense extract for 48 hr. Control wells were treated with equivalent amount of medium alone. Green live cells showed normal morphology; yellow early apoptotic cells showed nuclear margination and chromatin condensation.

CONCLUSIONS

T. pratense extract showed anti-angiogenesis properties. It is potentially beneficial for further development of new anti-cancer agents. The present data open a new possible approach to the treat of cancer. Future studies are recommended to seek if *T. pratense* provides useful results in *in vivo* models.

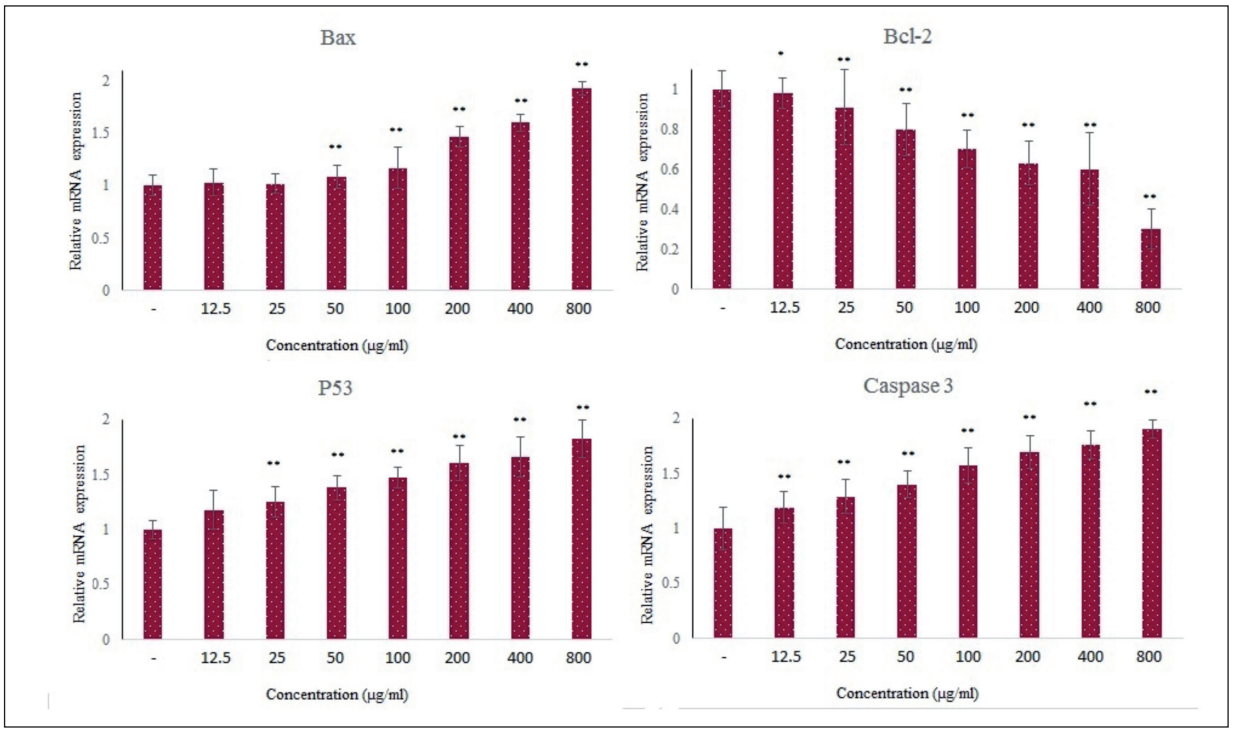

Fig. 5. Expression level of P53 (tumor suppressor), Bax (pro-apoptotic), Bcl-2 (anti-apoptotic) and Caspase-3 (required enzyme for execution of apoptosis) genes in HUVEC cells after treatment with different concentration of Trifolium pretense extract for 48 hr was evaluated by Real-time PCR. The data are expressed in terms of percent of control cells as the means ± SD. (*p<0.05; ** p<0.01 compared with control).

Acknowledgments:

This study was originated from a research grant 97320. The authors declare that there is no any conflict of interest in this study.

Funding:

This work was financially supported by the Vice-Chancellor for Research of Kermanshah University of Medical Sciences [Grant No.: 97320].

Conflict of Interest:

The Authors declare that they have no conflict of interests.

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