

INDUCTION OF APOPTOSIS AND INHIBITION OF AUTOPHAGY CELL DEATH IN THE HUMAN PROSTATE CANCER CELL LINES BY *TRIFOLIUM PRATENS* L. HYDROALCOHOLIC EXTRACT

M. KHAZAEI, M. PAZHOUHI

Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract – Objective: Prostate cancer is one of the most lethal forms of human cancer and paclitaxel is currently part of standard treatment for it. Combination therapy can enhance the anti-cancer activity of chemotherapy agents. *Trifolium pratense* L. has been suggested for cancer treatment in traditional medicine. In this study the effect of *T. pratense* hydroalcoholic extract on two prostate cell lines and normal fibroblast cells was investigated.

Materials and Methods: In this in vitro study, cells were treated with *T. pratense* extract and viability was evaluated using trypan blue staining, MTT assay, and lactate dehydrogenase activity measurement. Apoptosis and autophagy cell death were detected and quantified by fluorescent dyes staining. Nitric oxide production was measured using Griess reaction. Changes in expression level of some apoptotic and autophagic-related genes were investigated using Real-time PCR. The combination effect of extract and paclitaxel was evaluated by calculating the combination index and dose reduction index values.

Results: After treatment with *T. pratense* extract, the cell viability was significantly reduced in a time- and dose-dependent manner ($p < 0.05$). The effect of extract on normal cells was significantly smaller than cancer cells ($p > 0.05$). Apoptosis and autophagy cell death were significantly increased ($p < 0.05$). Also, *T. pratense* extract significantly decreased NO production ($p < 0.05$) by cancer cells. Combination of paclitaxel and *T. pratense* extract had a synergistic cytotoxic effect.

Conclusions: *T. pratense* showed an anti-cancer property via induction of apoptosis and autophagy cell death in prostate cancer cell lines.

KEYWORDS: Apoptosis, Autophagy, Prostate cancer, Cell culture, *Trifolium pratense* L.

LIST OF ABBREVIATIONS: AO: Acridine orange, AVO: Acidic vesicular organelles, CI: Combination index, DMEM/F12: Dulbecco's modified eagle medium/Ham's F12 nutrient mixture, DRI: Dose reduction index, EB: Ethidium bromide, FBS: Fetal bovine serum, LDH: Lactate dehydrogenase, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NO: Nitric oxide, PI: Propidium iodide, RPMI: Roswell Park Memorial Institute.

INTRODUCTION

Prostate cancer is one of the most common cancers and the second leading cause of cancer-related death among men. It accounts for 33% of all newly diagnosed malignancies, and usually develops over 50 years of age^{1,2}. Recently, standard treatment for prostate cancer consists of surgery (radical prostatectomy), radiotherapy (external-beam radiotherapy and/or brachytherapy) and chemotherapy. Paclitaxel

(Taxol), classified with the taxane group, is used as a chemotherapy agent in the treatment of prostate cancer. It is a mitotic inhibitor that binds to tubulin and inhibits the disassembly of microtubules, thereby resulting in the inhibition of cell proliferation. This agent also induces apoptosis by binding to and blocking the function of the anti-apoptotic protein Bcl-2³. Although these methods are initially useful for the majority of patients, but acquired drug resistance is the main problem in successful therapy



of patients and tumors become more aggressive and unresponsive to standard treatment. Furthermore, like most of the chemotherapy agents, paclitaxel has different side-effect profiles⁴. So there is an urgent need for safe and effective therapeutic approaches for prostate cancer. Currently, there is growing interest in developing combination therapy using multiple anti-cancer agents as a new strategy to overcome drug resistance. Different anti-cancer agents affect different targets and cell subpopulations and therefore, can enhance the therapeutic effects, reduce dose and side effects and prevent or delay the induction of drug resistance⁵. Several studies have suggested that consumption of a plant-based diet have a useful effect on the cancer therapy. Over 60% of approved and currently used chemotherapeutic drugs have been isolated from natural products, mostly of plant origin⁶. Some diet and nutritional supplementation have often been analyzed by several studies for the beneficial effect on prostate cancer^{7,8}. *Trifolium pratense* L., a member of *Leguminosae* or *Fabaceae* family, is a short-lived biennial flowering plant, which has been used as a health food for humans. It is found natively in Europe, Western Asia, and northwest Africa, but it has been naturalized in other continents, like North and South America. Nowadays, many isoflavone preparations derived from *T. pratense* are available as nutritional supplements⁹. It has also been used in traditional medicine to treat whooping cough, asthma, eczema and eye diseases¹⁰. *T. pratense* has also been suggested for cancer treatment in traditional medicine book. Our previous work showed the anti-cancer properties of this extract on glioma cell line (U87MG)¹⁰. The aim of the present study was to determine the effect of *T. pratense* hydroalcoholic extract on two human prostate cancer cell lines and normal fibroblast cells.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The human prostate cancer cell lines (LNCap and PC3) were obtained from the National Cell Bank of Iran. Fibroblasts isolated from the skin in primary culture were kindly supplied by Dr. Kamran Mansouri (Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.) and used between passages 3 and 6. Paclitaxel, trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Dulbecco's modified eagle medium/Ham's

F12 nutrient mixture (DMEM/F12), Roswell Park Memorial Institute (RPMI)-1640 Medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). All experiments were performed in triplicate and repeated independently at least three times.

COLLECTION AND EXTRACTION OF THE PLANT MATERIAL

T. pratense seed was prepared and cultured in a farm and the herbs were collected during May 2017. The species of plant were confirmed by a taxonomist from Kermanshah University of Medical Sciences (Kermanshah, Iran). Next, the aerial part of herbs was collected, shade-dried at room temperature and powdered. Then, 15 g of powder was dissolved in 150 mL of 70% ethanol for 48 h in darkness. It was subsequently filtered through filter paper and dried to evaporate the alcohol. The solvent of the final powder was serum-free cell culture medium, and the prepared concentrations were passed through a 0.22 μm filter before use¹¹.

CELL CULTURE AND TREATMENT

Both cell lines were seeded in cell culture flasks containing Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. The medium of normal fibroblast cells was DMEM/F12. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Following reaching to confluency, the cells were detached with 0.25% trypsin/EDTA solution, centrifuged at 1500 rpm for 5 minutes, and counted with a hemocytometer. Approximately 1.5 \times 10⁴ and 7 \times 10⁴ cells per well were seeded in 96 and 24 well plates, respectively. Paclitaxel were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and stored at -20°C until use. The cells were treated with 12.5, 25, 50, 100, 200, 400 and 800 $\mu\text{g}/\text{mL}$ of *T. pratense* extract.

DYE EXCLUSION TEST

Trypan blue staining is used to determine the viability of cells. This method is based on the ability of the live cells with intact membranes to exclude certain dyes, such as trypan blue. The cells were cultured in 24-well plates and after 24 hr, the culture medium was replaced with new serum-free medium containing various concentration of *T. pratense* extract. Plates were incubated for 24, 48 and 72 hr. Subsequently, the cells were trypsinized and the suspension was mixed with an equal volume of 0.4% trypan blue solution. The number of dead cells (have a blue cytoplasm) vs. the total number of cells was calculated as the percentage of viability¹².

MTT ASSAY

MTT staining is a standard colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a blue formazan crystal by mitochondrial succinate dehydrogenase enzyme. Briefly, cancer cells were seeded in 96-well culture plates. After incubating the plates for 24 hr at 37°C in a humidified incubator, the cells were treated with various concentrations of *T. pratense* for 24, 48 and 72 hr or left untreated in the control group. Next, the culture medium was removed and 30 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C. After 4 hr the formazan product was dissolved in 100 µl DMSO and the optical density (OD) of each sample was measured using an ELISA reader at 570 and 680 nm. The percentage of cell viability was calculated according to the following equation¹³:

$$\text{Cell viability (\%)} = \left[\frac{\text{OD}_{570, 630} (\text{sample})}{\text{OD}_{570, 630} (\text{control})} \right] \times 100.$$

The IC₅₀ values of *T. pratense* extract against two-cancer cell lines were calculated using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

**LACTATE DEHYDROGENASE
CYTOTOXICITY (LDH) ASSAY**

LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serve as a general means to assess cell viability. Briefly, cancer cells were cultured in 24-well plates and the plates were incubated overnight at 37°C. The culture medium (300 µl) containing various concentration of extract was added to each well, and the plates were incubated for 24, 48 and 72 hr. Then, 100 µl of medium from each well was transferred to new plates. LDH activity was measured using Cytotoxicity Detection Kit (Roche Chemical Co., Basel, Switzerland) according to the manufacturer's procedure. Finally, the OD at 490 nm with a reference wavelength of 690 nm for each sample was measured¹⁴.

TUNEL ASSAY

Apoptosis was evaluated by labeling the 3'-hydroxyl termini in the DNA fragments using an *In Situ* Cell Death Detection Kit, AP (Roche Diagnostics, Basel, Switzerland) and according to the manufacturer's instructions. Briefly, after 48 hr treatment with *T. pratense* extract in a 96 well plate, the cells were fixed using 4% paraformaldehyde in PBS (freshly prepared) for 1 hr, permeabilized using a solution (0.1% Triton X-100, 0.1% sodium citrate) for 5 min on ice, and incubated with 50 µl of TUNEL mixture solution (label and enzyme solution) at 37°C for 1 hr in a humidified incubator.

For differential staining of the cells the PI staining solution was added and the plate was incubated for 4 min at room temperature. Finally, cells were analyzed using a fluorescence microscope. All the mentioned stages are performed in dark condition. The apoptotic index of the cells was calculated as follow¹⁵: Apoptotic index (%) = (number of apoptotic cells/total number of cells) × 100

AO/EB DOUBLE STAINING

To observation of the live, early and late apoptotic, and necrotic cells under the fluorescent microscope, AO/EB double staining was performed. AO permeates all cells and makes the nuclei appear green. EB is only taken up by cells when cytoplasmic membrane integrity is lost, and stains the nucleus red. EB also dominates over AO. Thus, live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus. The cells were seeded in 24-well plates and incubated overnight in a humidified 5% CO₂ incubator at 37°C for 24 hr. Then, cells were treated extract. After 48 hr, wells were washed with PBS and stained using AO/EB dye mix (1 part of 100 µg/ml of AO and 1 part of 100 µg/ml of EB in PBS). The live, apoptotic and necrotic cells were observed under the fluorescent microscope¹⁶.

**DETECTION OF ACIDIC VESICULAR
ORGANELLES (AVOs)**

To analyze the autophagy induction, AVOs, which consist predominantly of autophagosomes, and autolysosomes, were quantified using AO staining. AO accumulates in acidic organelles in a pH-dependent manner in cells. At neutral pH, it emits green fluorescence, but within acidic environments becomes protonated and gets trapped within the organelle and then aggregates and emits red fluorescence. Cancer cells were seeded in a 24-well plate and treated with various concentrations of extracts. After 48 hr, the cells were stained with AO (1 µg/mL) for 20 min in dark condition. After washing out the excess dye with PBS, the morphological change was evaluated using a fluorescence microscope. The percentage of autophagic cells was calculated as follows¹⁷: Autophagic cells (%) = (the number of cells with AVOs / the total number of stained cells) × 100.

NITRIC OXIDE (NO) MEASUREMENT

The effect of *T. pratense* on NO production by prostate cancer cells was investigated by colorimetric Griess reaction. This reaction is widely used to determine total nitrate and nitrite concentration as an index of NO production in biological samples. After treatment with different concentration of extract for



TABLE 1. The primer sequences of key genes.

Sequence	Genes
Caspase-3	Forward: 5'-CAAACCTTTTTCAGAGGGGATCG-3' Reverse: 5'-GCATACTGTTTCAGCATGGCAC-3'
Caspase-8	Forward: 5'-GGATGGCCACTGTGAATAACTG-3' Reverse: 5'-TCGAGGACATCGCTCTCTCA-3'
Caspase-9	Forward: 5'-TGTCCTACTCTACTTTCCAGGTTT-3' Reverse: 5'-GTGAGCCCACTGCTCAAAGAT-3'
P53	Forward: 5'-TAACAGTTCCTGCATGGGCGGC-3' Reverse: 5'-AGGACAGGCACAAACACGCACC-3'
Bcl-2	Forward: 5'-TTGTGGCCTTCTTTGAGTTCGGTG-3' Reverse: 5'-GGTGCCGTTTCAGGTACTCAGTCA-3'
Bax	Forward: 5'-CCTGTGCACCAAGGTGCCGGAAC-3' Reverse: 5'-CCACCCTGGTCTTGGATCCAGCCC-3'
Beclin-1	Forward: 5'-GCCGAAGACTGAAGGTCA-3' Reverse: 5'-GTCTGGGCATAACGCATC-3'
LC3	Forward: 5'-GATGTCCGACTTATTCGAGAGC-3' Reverse: 5'-TTGAGCTGTAAGCGCCTTCTA-3'
ATG-7	Forward: 5'-ATTGCTGCATCAAGAAACCC-3' Reverse: 5'-GATGGAGAGCTCCTCAGCA-3'
GAPDH	Forward: 5'-TCCCTGAGCTGAACGGGAAG-3' Reverse: 5'-GGAGGAGTGGGTGTCGCTGT-3'

48 hr, 400 µl of cell culture supernatant from each group was collected. In order to remove the proteins, 6 mg of zinc sulfate was added to each sample and centrifuged at 10000 g for 10 minutes at 4°C. Then, 100 µl of each deproteinized sample was transferred to microplate wells and 100 µl vanadium (III) chloride, 50 µl 2% sulfanilamide (in 5% HCl) and 50 µl 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (in deionized water) were added, respectively. After incubation at room temperature for 30 min, the absorbance was measured by a microplate reader at 540 nm and 630 nm. The NO concentrations were calculated on the basis of a NaNO₂ standard curve¹⁸.

PACLITAXEL AND *T. PRATENSE* EXTRACT COMBINATION TREATMENT

The cell lines were treated with paclitaxel for 48 hr in 96 well plates and MTT assay was performed as described above. The IC₅₀ values of chemotherapy agent against two cancer cell lines were calculated using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The combination of paclitaxel and *T. pratense* extract was prepared in constant concentration ratio (1:31 for LNCap and 1:64 for PC3) based on their corresponding IC₅₀ values in serial dilutions above and below the IC₅₀ value of each agent, and then the MTT assay was performed again¹⁹.

MEDIAN EFFECT ANALYSIS

To determine and quantify the nature of paclitaxel and *T. pratense* extract interaction (synergistic, additive, or antagonistic) in combination treatment, the combination index (CI) and dose reduction index (DRI) were

calculated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). The CI values were interpreted as additive (CI = 1), synergistic (CI < 1) and antagonistic (CI > 1). The DRI values represent the degree to which the concentration of a compound can be reduced when used in combination with another compound to maintain an equivalent effect, and Fa is the fraction of cell death ranging from 0 (no cell killing) to 1 (100% of cell killing)¹⁹.

REAL-TIME PCR

The effects of various concentration of *T. pratense* extract on the expression level of some apoptotic and autophagic-related genes were analyzed by real-time PCR. Total RNA from cancer cells, treated with *T. pratense* extract for 48 hr, was prepared by total RNA isolation kit (DENAzist, Tehran, Iran) and complementary DNA (cDNA) synthesis was carried out using cDNA synthesis kit (Vivantis Technologies, Selangor DE, Malaysia). Real-time PCR was performed using SYBR Premix Ex Taq technology (TaKaRa Bio Inc., Otsu, Shiga, Japan) on the Applied Biosystems StepOne Real Time PCR System. 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 (2^{-ΔΔCt}) method. The primer sequences were as follows:

STATISTICAL ANALYSIS

All data are presented as mean ± standard deviation of three independent experiments. Statistical evaluation was done using one-way analysis of variance

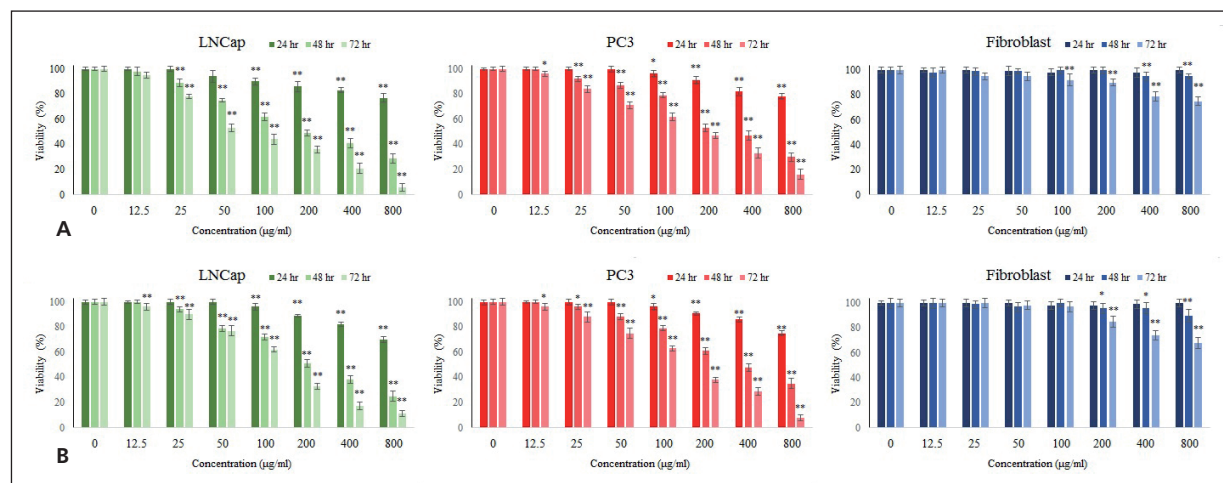


Fig. 1. The effect of *Trifolium pratense* on proliferation of LNCap, PC3 and human fibroblast cells. Cells were treated with indicated concentrations of extract for 24, 48 and 72 hr. Viability was determined using (A) MTT assay and (B) trypan blue staining as described in the methods section. Control wells were treated with equivalent amount of medium alone. The data are expressed in terms of percentage of control cells as the means \pm SD (* p <0.05; ** p <0.01 compared with control).

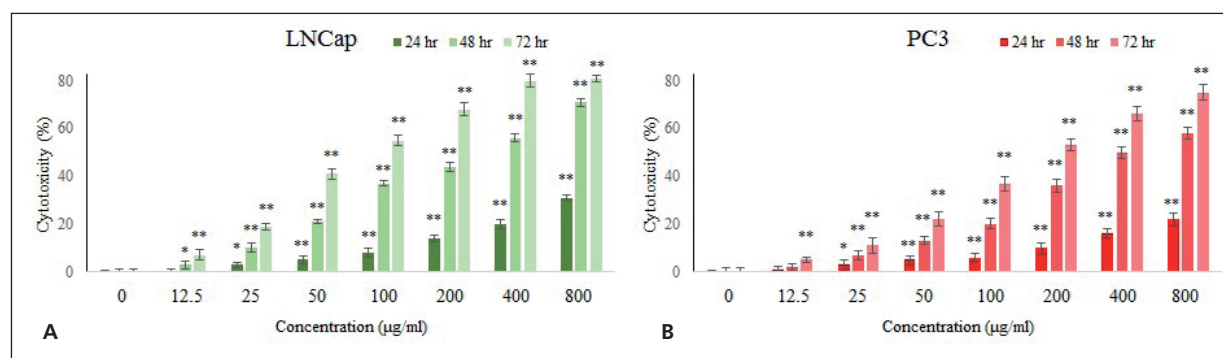


Fig. 2. The effect of *Trifolium pratense* on LDH release from LNCap and PC3 cells. Cells were treated with extract for 24, 48 and 72 hr. Control wells were treated with equivalent amount of medium alone. The data are expressed in terms of percentage of control cells as the means \pm SD. (* p <0.05; ** p <0.01 compared with control).

with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software, and differences were considered to be statistically significant when $p < 0.05$.

RESULTS

T. PRATENSE EFFECT ON CELL VIABILITY

The cytotoxic effect of the extract was evaluated on LNCap, PC3 and normal fibroblast cells using the MTT and trypan blue assays (Figure 1 A and B). The half-maximal inhibitory concentration (IC_{50}) of the extract was estimated using GraphPad Prism 5 soft-

TABLE 2. IC_{50} values for *Trifolium pratense* in the two prostate cancer cell lines.

	24 hr	48 hr	72 hr
LNCap	2196.44	234.42	93.43
PC3	2537.64	321.71	152.48
Fibroblast	169915.05	73415.56	1790.07

ware (GraphPad Software Inc., La Jolla, CA, USA). Table 2 depicts IC_{50} values for the 24, 48, and 72 hr treatments. The results showed that all the cell lines responded to the cytotoxic effect of the plant extract in a dose- and time-dependent manner. The LNCap cancer cells, however, were more sensitive to the extract as shown by its IC_{50} values for 24, 48, and 72 hr. Moreover, the extract exhibited selective cytotoxicity in normal fibroblast cells with higher IC_{50} values. The anti-proliferative activity of the extract was further evaluated using the LDH measurement assay. This assay shows the integrity of cell plasma membrane. As presented in Figure 2, the LDH activity in cell culture medium increased with increasing concentration of the extract. So, cytotoxicity of extract is accomplished with plasma membrane damage.

T. PRATENSE EFFECT ON APOPTOSIS

The apoptotic effect of the hydro-alcoholic extract of *T. pratense* was explored by TUNEL assay and staining the cancer cells with Ao/Eb dyes.



The results of TUNEL assay showed that the observed anti-proliferative effects occur through the mechanisms associated with apoptosis. The extract induced apoptosis in a concentration-dependent manner (Figure 3). The LNCap cell line was more sensitive to the extract when compared with PC3 cell line. The apoptosis-inducing potential of the extract was further tested in cells using the Ao/Eb double staining assay. The typical morphological changes in apoptotic cells including cell shrinkage,

chromatin condensation and nuclear fragmentation were showed in Figure 4.

For LNCap cell line live cells with normal morphology were abundant in the control group and 12.5 $\mu\text{g/ml}$, whereas early apoptotic cells occurred in cells treated with 25 and 50 $\mu\text{g/ml}$, both early and late apoptotic cells were observed in cells treated with 100, 200 and 400 $\mu\text{g/ml}$, in and 800 $\mu\text{g/ml}$ group all of the cells were in late stage. In PC3, live cells with normal morphology were observed

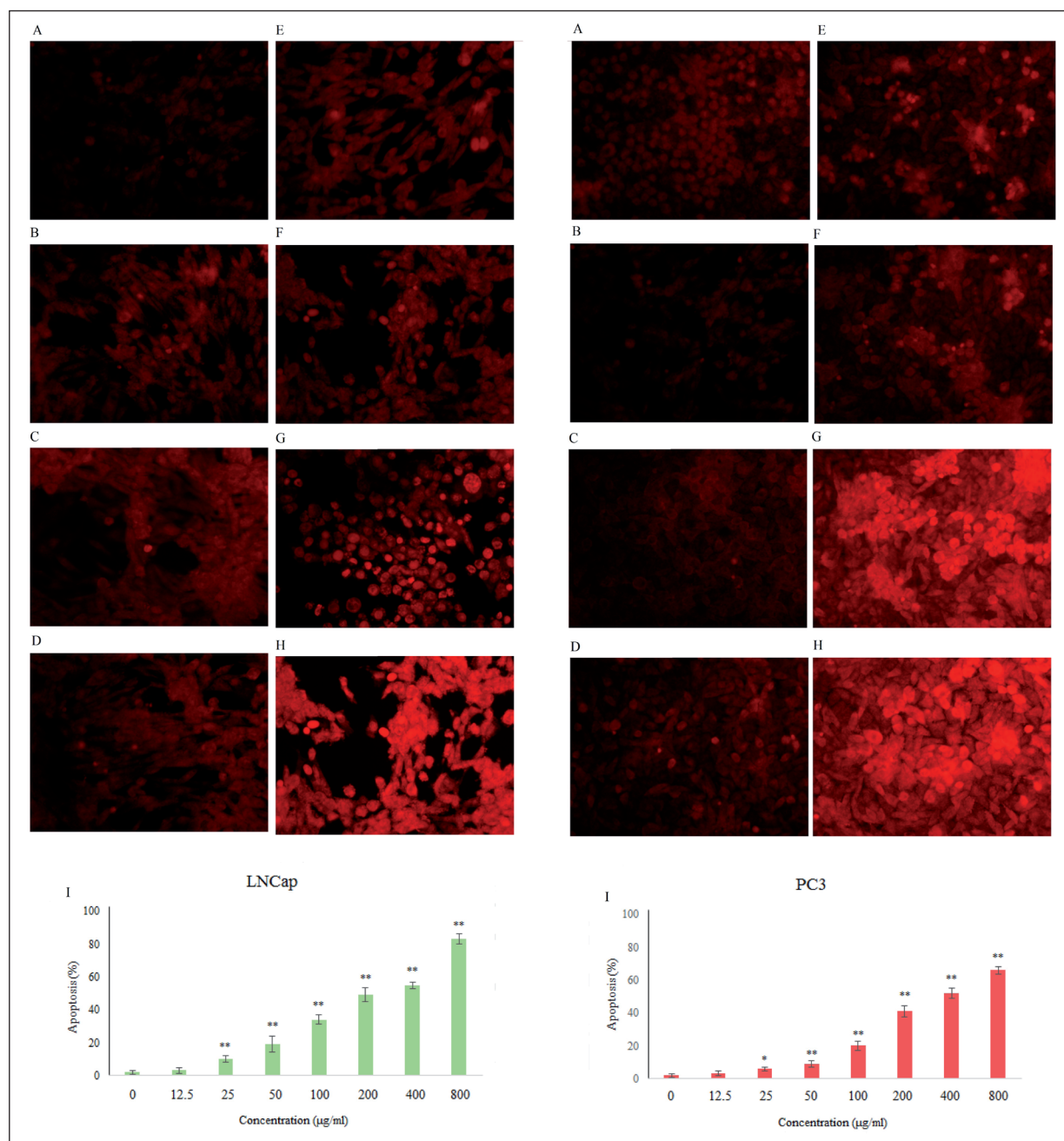


Fig. 3. The effect of *Trifolium pratense* on apoptotic potential in LNCap and PC3 cells was evaluated using TUNEL staining. *A*, Control group; *B*, In the presence of 15.62; *C*, 31.25; *D*, 62.5; *E*, 125; *F*, 250; *G*, 500; *H*, 1000 $\mu\text{g/ml}$ of *T. pratense* extract; and *I*) columns mean percentage of apoptotic cells from three independent experiments performed in triplicate. Control cells treated with serum free medium. *p*-values were determined using one-way-ANOVA (**p*<0.05 compared with control; ***p*<0.01 compared with control).

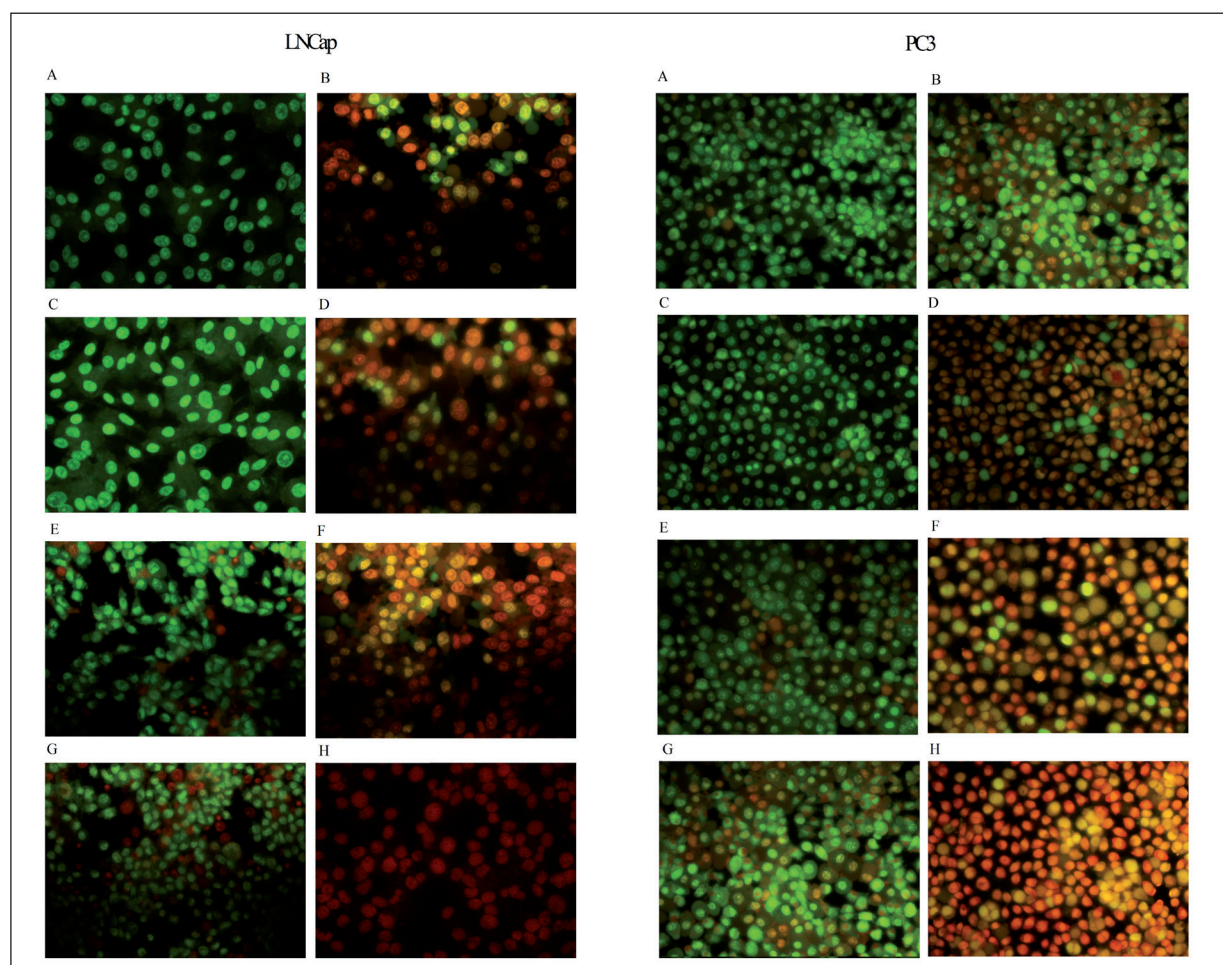


Fig. 4. LNCap and PC3 cells were stained by AO/EB and observed under fluorescence microscope: *A*, Control group; *B*, in the presence of 15.62; *C*, 31.25; *D*, 62.5; *E*, 125; *F*, 250; *G*, 500; and *H*, 1000 µg/ml of *Trifolium pratense* extract. Control wells were treated with equivalent amount of medium alone. Green live cells showed normal morphology; yellow early apoptotic cells showed nuclear margination and chromatin condensation. Late orange apoptotic cells showed fragmented chromatin and apoptotic bodies

in the control group, whereas early apoptotic cells occurred in the group treated with 25 and 50 µg/ml, early and late apoptotic cells were observed in 100, 200 and 400 µg/ml and in the presence of 800 µg/ml most of cells were in late stage (Figure 4). Necrosis was negligible in all tested groups. These morphological changes suggest that apoptosis increased in cells treated with *T. pratense* extract in dose- dependent manner in both cell lines.

T. PRATENSE EFFECT ON AUTOPHAGY

The effect of the plant extract on autophagy cell death was also investigated by staining the cells with Ao dye and a fluorescent microscope. The results showed that *T. pratense* extract induced cell death through the activation of autophagy. The extract induced autophagy in a concentration-dependent manner (Figure 5). The LNCap cell line was a bit sensitive to the extract when compared with other cell line.

T. PRATENSE EFFECT ON NO PRODUCTION

NO concentration in cell culture medium was assessed by Griess assay. The effect of different concentrations of *T. pratense* extract on prostate cancer cells after 48 hr treatment indicated a dose-dependent decrease in NO production for both cell lines. The difference compared to the control group was significant with the 25, 50, 100, 200, 400 and 800 µg/mL doses for LNCap and PC3 (Figure 6).

T. PRATENSE EFFECT IN CO-TREATMENT WITH PACLITAXEL

The effect of paclitaxel on cell viability was evaluated by MTT assay for both cell lines (Figure 7 A). Paclitaxel showed cytotoxic effect in a dose-dependent manner in both cell lines. The IC₅₀ values of paclitaxel were 6.34 nM and 5.36 nM for LNCap and PC3, respectively. Then, cancer cells were treated with combination of paclitaxel and *T. pratense* extract for 48 hr. Reduction in the cell viability by

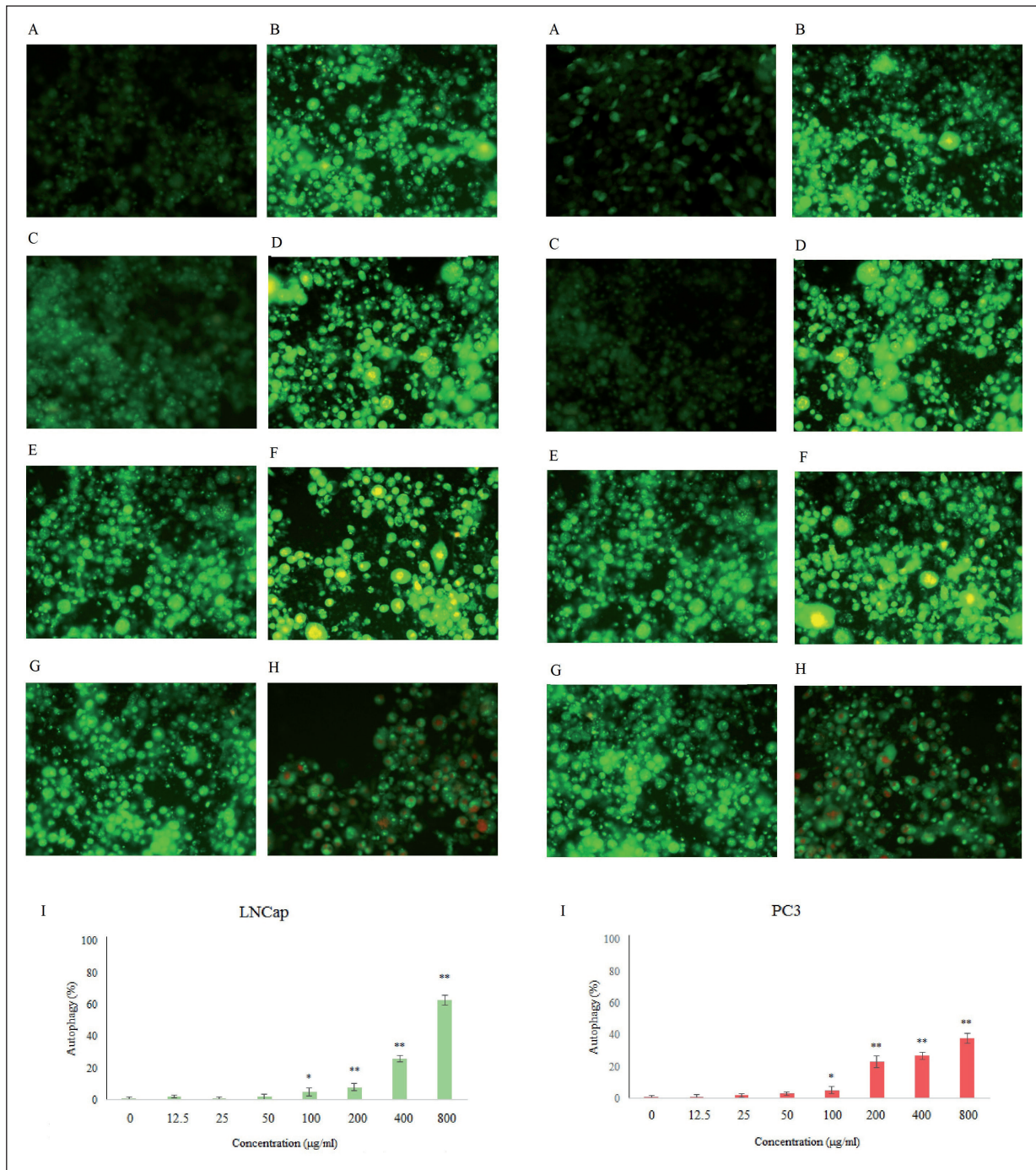
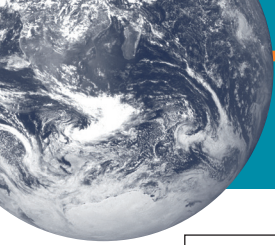


Fig. 5. The effect of *Trifolium* pretense on autophagy was monitored by AO staining in LNCap and PC3 cells. (A) Control group (B) in the presence of 15.62; C, 31.25; D, 62.5; E, 125; F, 250; G, 500; H, 1000 µg/ml of extract and (I) columns mean percentage of autophagic cells from three independent experiments. Control wells were treated with equivalent amount of medium alone. Red dots indicate autophagic vesicles. p-values were determined using one-way-ANOVA (* $p < 0.05$ compared with control; ** $p < 0.01$ compared with control).

paclitaxel and *T. pratense* extract combination was greater than either each one alone (Figure 7 B). In addition, CI plot (Figure 7 C], DRI plot (Figure 7 D), and the isobologram (Figure 7 E) were also presented. The results showed that the CI values in all combinations were smaller than 1, implying a synergistic effect in all combination tests. The

DRI values for paclitaxel were >1 indicating a dose reduction for paclitaxel in a given therapeutic effect.

T. PRATENSE EFFECT ON APOPTOTIC AND AUTOPHAGIC-RELATED GENES EXPRESSION

The expressions of some apoptotic and autophagic-related genes were evaluated using Real-time

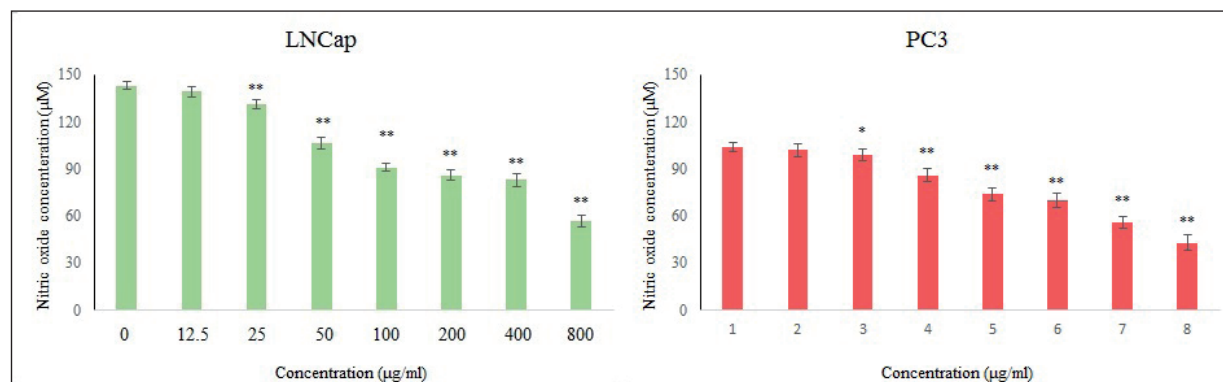


Fig. 6. The effect of *Trifolium pratense* on NO production by LNCap and PC3 cells was measured by Griess reaction. Control wells were treated with equivalent amount of medium alone. The data are expressed in terms of percent of control cells as the means \pm SD. (* p <0.05; ** p <.01 compared with control).

PCR. As shown in Figure 8, p53 was upregulated in cells that were treated with *T. pratense* extract. Results of Real-time PCR also suggested a down-regulation of Bcl-2 and up-regulation of Bax mRNA expression after 48 hr treatment with *T. pratense* extract. Exposure of cells to *T. pratense* extract led to increased mRNA expression of caspase-3, 8 and 9 genes. Also the extract increased the LC3, beclin-1 and ATG-7 mRNA levels. Thus, *T. pratense* extract induced apoptotic and autophagic cell death in prostate cancer cells at the transcriptional level.

DISCUSSION

Cancer, one of the leading causes of death worldwide, is the result of multiple alterations in cellular signaling pathways²⁰. Common chemotherapy is often accompanied by various side effects on normal tissues and cells and gradual drug resistance in cancer cells²¹. The use of plants as a source of human therapeutic medicine has a long history. Nowadays, numerous studies have reported the potential of some plants in stopping or slowing growth in cancer cells without significant adverse effect on normal cells²². The mechanisms of the plants for anti-cancer properties are numerous and most of them cause apoptotic cell death induction via intrinsic or extrinsic, and caspase and/or p53-dependent or independent mechanisms. Also anti-cancer potential of some plant is reported through induction of autophagy, necrosis-like programmed cell death, mitotic catastrophe, and senescence²³. *T. Pratense* is used as a flavor to make a sweet-tasting herbal tea. In the traditional medicine of India, *T. pratense* is used as deobstruent, antispasmodic, expectorant, sedative, anti-inflammatory, and anti-dermatosis agent. In alternative medicine, it is suggested as a treatment for a variety of human disorders, including coughs,

disorders of the lymphatic system and a variety of cancers. Some studies proposed that due to its activity on estrogen receptors, it is contraindicated in people with a history of breast cancer, endometriosis, ovarian cancer, uterine cancer, uterine fibroids, or other estrogen-sensitive conditions, but others have suggested the high isoflavone content counteracts this and even provides benefits in these conditions²⁴. The present study evaluated the anti-cancer activity of the *T. pratense* hydro-alcoholic extract on prostate cancer cells (LNCap and PC3) and normal human fibroblast cells. The results of this study showed that the extract had a cytotoxic effect towards both cancer cell lines in a dose- and time-dependent manner. The obtained IC₅₀ values demonstrated that LNCap cells are more sensitive to the cytotoxic activity of the plant while normal human fibroblast cells, showed less sensitivity to the extract. The potential of the anti-cancer agent to distinguish between normal and cancer cells is an important paradigm in the design and discovery of new chemotherapeutic agents. Consistent with this concept, trypan blue dye exclusion and LDH assays confirm the anti-neoplastic activities of the extract against cancer cell lines compared to the normal human fibroblast cells. *T. pratense* is known to be rich in several phytochemicals like isoflavones, flavonoids, pterocarpan, coumarins and tyramine²⁵. Its main isoflavones are biochanin A, formononetin, daidzein, genistein, pratensein, prunetin, pseudobaptigenin, calycosin, methylroborol, afrormosin, texasin, irilin B and irilone²⁶. Some of these phytochemicals have been reported to possess anti-neoplastic activities against different cancer cell lines. Dietary flavonoids are the most abundant polyphenols in plant sources and their activities against various cancers have also been reported. Several plant-derived flavonoids (silymarin, genistein, quercetin, daidzein, luteolin, kaempferol, apigenin, and epigallocatechin 3-gallate)

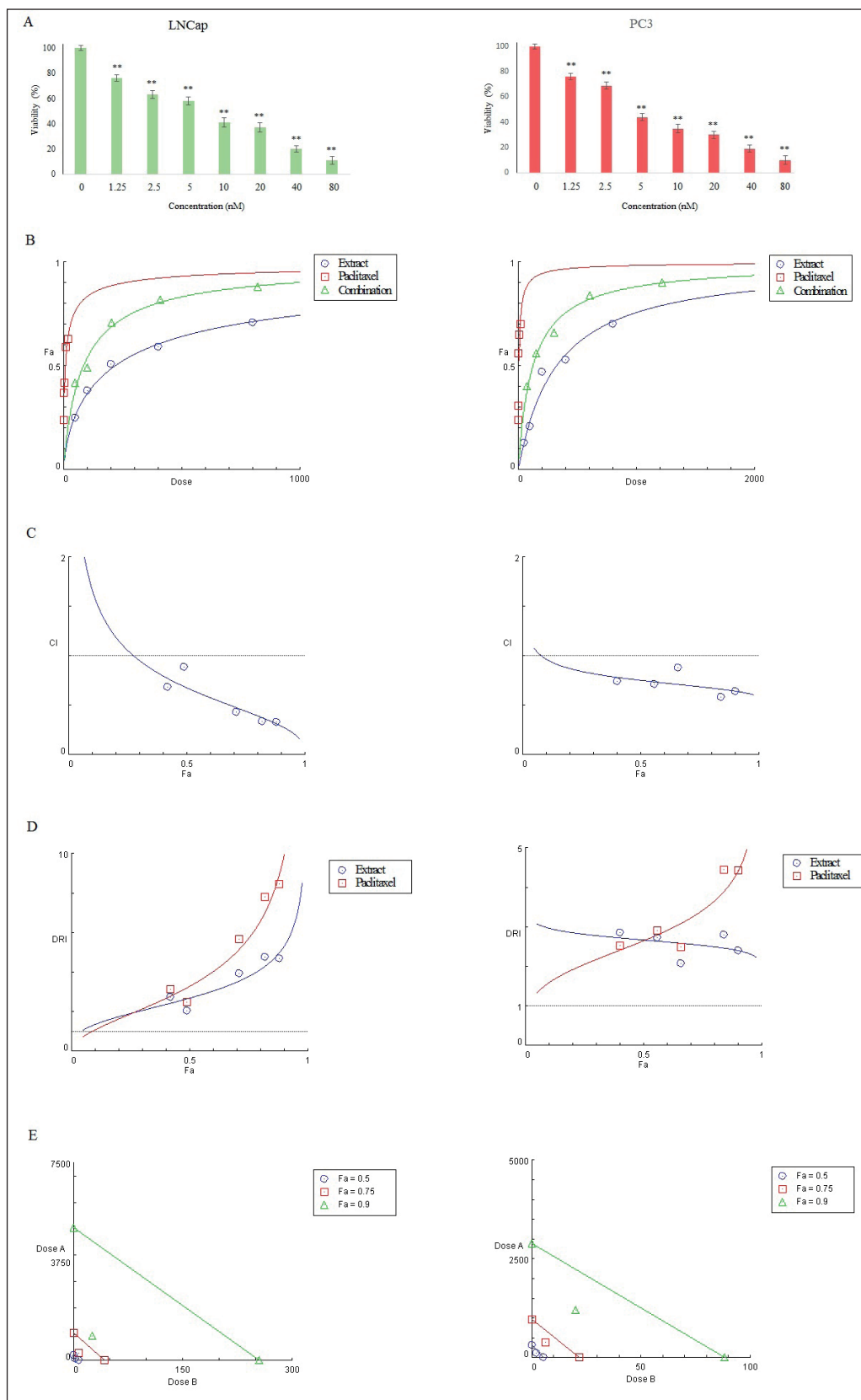


Fig. 7. *A*, The effect of paclitaxel on proliferation of LNCap and PC3 cells. *B*, Dose- effect curves for paclitaxel, Trifolium peratense extract, and their combination after 48 hr treatment. *C*, Combination index plot: The combination index is plotted as a function of Fa. *D*, Dose reduction index plot for combination: Dose reduction index values at different Fa values for each drug in the combination. *E*, Isobologram for combination: classic isobologram at IC50, IC75, and IC90. Cells were treated with indicated concentrations of for 48 hr. The data are expressed in terms of percent of control cells as the means \pm SD. (* p <0.05; ** p <0.01 compared with control).

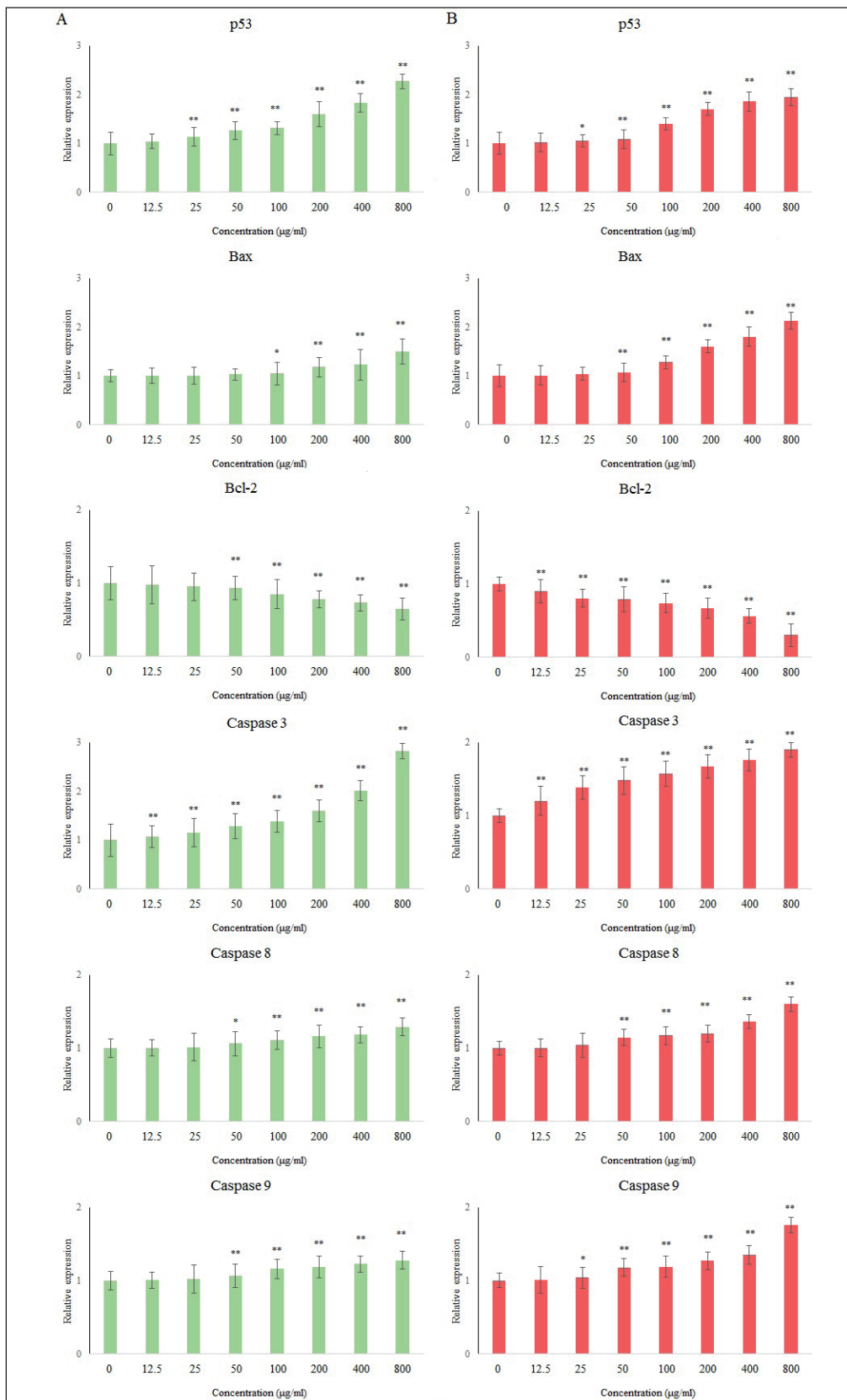


Fig. 8. Expression level of p53 (tumor suppressor), Bax (pro-apoptotic), Bcl-2 (anti-apoptotic), caspase-3 (required enzyme for execution of apoptosis), caspase-8 (mediator of extrinsic pathway) and caspase 9 (mediator of intrinsic pathway) genes in LNCap and PC3 cells after treatment with different 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 \pm SD. (* $p < 0.05$; ** $p < 0.01$ compared with control).

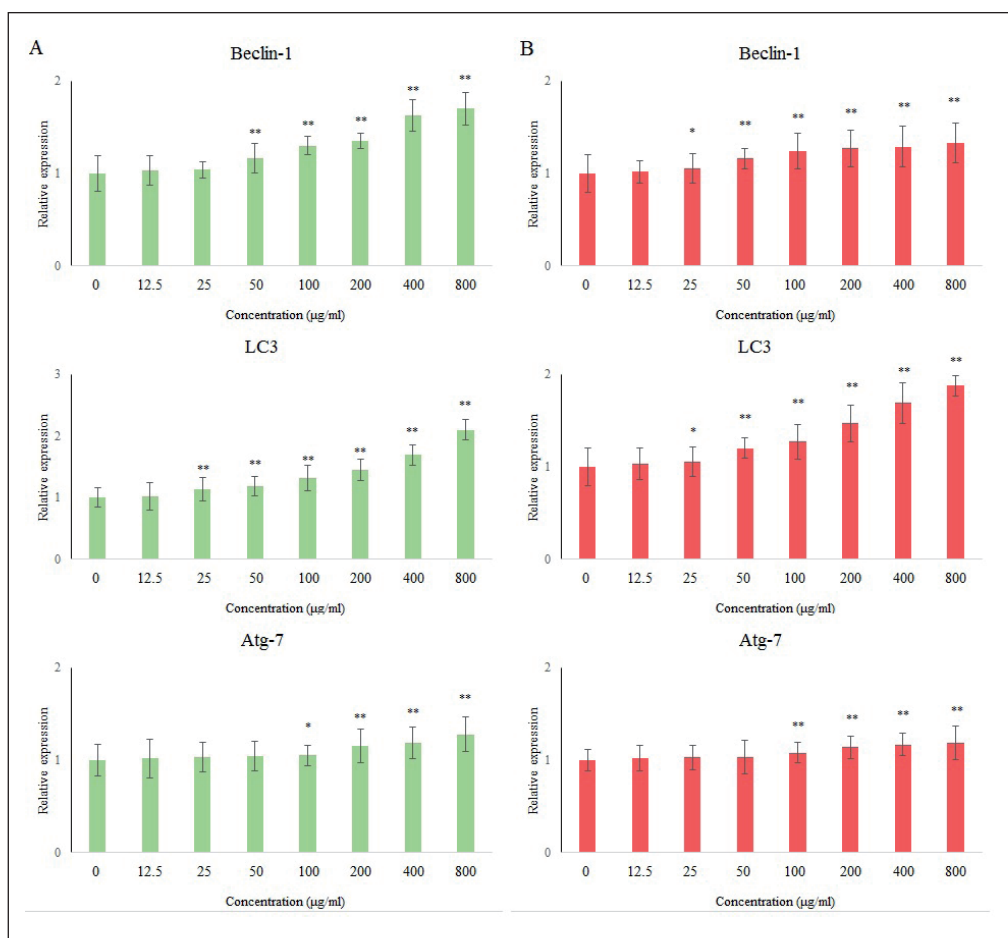


Fig. 9. Expression level of beclin-1 (key positive autophagic regulator), ATG-7 (essential autophagy gene), and LC 3 (essential for autophagosome formation) in LNCap and PC3 cells after treatment with different concentration of Trifolium pretense for 48 hr was evaluated by real-time PCR. The data are expressed in terms of percent of control cells as the means \pm SD. (* p <0.05; ** p <0.01 compared with control).

have an anti-proliferative effect on some cancers such as prostate, colorectal, breast, thyroid, lung, and ovarian. Their anti-cancer effect is mediated by activation of apoptosis, cell cycle arrest at G1 or G2/M phase, inhibition of metabolizing enzymes, reactive oxygen species formation, vascular endothelial growth factor and basic fibroblast growth factor. Also, some flavonoids have been reported to reduce cancer cells drug resistance. Genistein and daidzein, two members of flavonoid family, have noticeable anti-proliferation effect against breast cancer, due to their structural similarity with estrogen. Anti-cancer effect quercetin, another member of flavonoid family, against colon cancer and glioma tumors, is mediated by activation of autophagy signaling pathway.

Nowadays, a variety of these flavonoids are used in dietary supplements, but none of them have been approved for clinical use²⁷. From pterocarpan family Indigo carpan, showed anti-proliferative activity in human cancer cell lines via induction of caspase dependent apoptosis pathway²⁸. Anti-cancer activ-

ity of coumarins is mediated by various pathways including inhibition of kinase, cell cycle progression, angiogenesis, heat shock protein (HSP90), telomerase, mitotic activity, carbonic anhydrase, monocarboxylate transporters, aromatase and sulfatase²⁹.

To further study about the pathways of the cell death induced by the extract, apoptosis and autophagy was evaluated using the TUNEL assay, Ao/Eb staining and AVO detection. DNA fragmentation is generally accepted as one of the biomarkers of apoptosis³⁰. The results showed the concentration-dependent apoptotic and autophagic-inducing potential of the extract. Here again LNCap cells were more sensitive to the extract compared to PC3 cell line. Unlike necrosis, apoptosis does not trigger an inflammatory response that destroys the normal cells in the surrounding microenvironment³¹. Cancer cells, in most cases, exhibit resistance to apoptosis in order to sustain their uncontrolled proliferation and, therefore, any apoptosis-inducing agent is desirable as a chemotherapeutic agent

against cancer³². Autophagy is a catabolic pathway in cells for the clearance of damaged organelles. It has also been considered as a cell death mechanism that could function as alternative forms of cell death when apoptosis is unavailable. As mentioned above, most of the cancer cells have defect apoptosis pathway and in this condition, autophagy could function as a backup for cell death³³. There are two main apoptotic pathways in cells; the extrinsic or death receptor mediated pathway and the intrinsic or mitochondrial mediated pathway. Caspase-3 is one of the most important effectors caspase that is involved in the final execution of both apoptosis pathway while caspase-9 and 8 are initiator caspases involved in the intrinsic and extrinsic pathways, respectively³⁴. To understand the mechanism of apoptosis induced by the *T. pratense* extract, caspase-3, caspase-9 and 8 mRNA expressions were evaluated. The results showed that the extract induced increased in caspase-3 expression in both cell lines. Measurement of caspase-9 and 8 expression showed a similar trend of increase in both cell lines. So, *T. pratense* extract induced apoptosis cell death through intrinsic and extrinsic pathways.

Several studies showed that the intensity of inducible nitric oxide synthase (iNOS) expression in the prostate tissue is correlated with the severity of tumor development and had prognostic values. iNOS expression and NO concentration generally contributes to tumor angiogenesis, tumor growth and metastasis, and tumor-related immune suppression. A study with prostate tumor spheroids showed that enhanced NO formation was associated with a decrease in tumor cell death³⁵. The results of this study showed that the extract decrease NO production in both cell lines. Nitric oxide also has a bifunctional regulatory effect on apoptosis. Inhibitory and inducing effect of NO on apoptotic signaling pathway have been reported in various *in vivo* and *in vitro* experimental models. NO can be an important endogenous inhibitor of apoptosis. Among the most important anti-apoptotic activities of NO are induction of cytoprotective stress proteins, cGMP-dependent inhibition of apoptotic signal transduction, suppression of caspase activity, and inhibition of cytochrome c release³⁶. Our data indicated that decrease in NO production by *T. pratense* extract may remove the anti-apoptotic effect of NO in prostate cancer cells.

We also explored whether *T. pratense* extract could demonstrate a therapeutically beneficial effect when administered in combination with paclitaxel or not. The results showed that extract enhanced the anti-proliferative activity of paclitaxel and combination treatment exerted synergistic cytotoxic effects on both cell lines with CI values between

0.33 and 0.89 for LNCap and 0.58 and 0.88 for PC3 cells. The mean CI of all tests is, 0.53 for LNCap and 0.71 for PC3. In other words, paclitaxel and *T. pratense* acted synergistically to reduce the viability of prostate cancer cells. This combination also results in a noticeable dose reduction for paclitaxel and reduces its IC50s to about 3.28 and 2.64 fold smaller against LNCap and PC3 cells, respectively. Paclitaxel, like many other chemotherapeutic drugs, has different types of side effects. Dose reduction of this drug for given therapeutic effect is clinically very important since this reduction leads to reduced general side effects of chemotherapy.

CONCLUSIONS

T. Pratens could be beneficial for further development of new chemotherapeutic agents. It also may be used as a dietary supplement for some patients who receive conventional chemotherapy with paclitaxel. The present data open a new possible approach in the treatment of prostate cancer. Investigation of the effect of *T. pratense* extract on radiotherapy response of prostate cancer cells is proposed. Future studies are necessary to seek if a combined treatment with *T. pratense* extract and paclitaxel afford a better result in *in vivo* models.

ACKNOWLEDGMENTS

The authors would like to thank the Fertility and Infertility Research Center and Kermanshah University of Medical Sciences.

FUNDING

This work was financially supported by the vice-chancellor for research of Kermanshah University of Medical Sciences [Grant No. 97319].

ETHICAL CONSIDERATION

There is no ethical consideration for this type of study. The study was approved by Ethical Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (IR. KUMS.REC.1397.268).

CONFLICT OF INTEREST

The Authors declare that they have no conflict of interests.

REFERENCES

- 1) Kus G, Kabadere S, Uyar R, Kutlu HM. Induction of apoptosis in prostate cancer cells by the novel ceramidase inhibitor ceranib-2. *In Vitro Cell Dev Biol Anim* 2015; 51: 1056-1063.
- 2) Rini BI, Small EJ. Prostate cancer update. *Curr Opin Oncol* 2002; 14: 286-291.
- 3) Roomi MW, Shanker N, Niedzwiecki A, Rath M. Induction of apoptosis in the human prostate cancer cell line du-145 by a novel micronutrient formulation. *Open Journal of Apoptosis* 2015; 4: 11-21.



- 4) Mukhtar E, Adhami VM, Mukhtar H. Targeting microtubules by natural agents for cancer therapy. *Mol Cancer Ther* 2014; 13: 275-284.
- 5) Bijnsdorp IV, Giovannetti E, Peters GJ. Analysis of drug interactions. *Methods Mol Biol* 2011; 731: 421-434.
- 6) Kinghorn AD, Chin YW, Swanson SM. Discovery of natural product anticancer agents from biodiverse organisms. *Curr Opin Drug Discov Devel* 2009; 12: 189-196.
- 7) Hori S, Butler E, McLoughlin J. Prostate cancer and diet: food for thought? *Br J Urol Int* 2011; 107: 1348-1359.
- 8) Willis MS, Wians FH. The role of nutrition in preventing prostate cancer: a review of the proposed mechanism of action of various dietary substances. *Clin Chim Acta* 2003; 330: 57-83.
- 9) Vlasisavljevic S, Kaurinovic B, Popovic M, Djurendic-Brenesel M, Vasiljevic B, Cvetkovic D, Vasiljevic S. *Trifolium pratense* L. as a potential natural antioxidant. *Molecules* 2014; 19: 713-725.
- 10) Booth NL, Overk CR, Yao P, Totura S, Deng Y, Hedayat AS, Bolton JL, Pauli GF, Farnsworth NR. Seasonal variation of red clover (*Trifolium pratense* L., Fabaceae) isoflavones and estrogenic activity. *J Agric Food Chem* 2006; 54: 1277-1282.
- 11) Khazaei M, Pazhouhi M, Khazaei S. Evaluation of hydro-alcoholic extract of *trifolium pratense* L. for its anti-cancer potential on U87MG Cell line. *Cell J (Yakhteh)* 2018; 20: 412-421.
- 12) Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* 2001; Appendix 3: Appendix 3B.
- 13) Riss TL, Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L. Editors In: Sittampalam GS, Coussens NP, Brimacombe K, Grossman A, Arkin M, Auld D, Austin C, Baell J, Bejcek B, Caaveiro JMM, Chung TDY, Dahlin JL, Devanaryan V, Foley TL, Glicksman M, Hall MD, Haas JV, Inglese J, Iversen PW, Kahl SD, Kales SC, Lal-Nag M, Li Z, McGee J, McManus O, Riss T, Trask OJ Jr., Weidner JR, Wildey MJ, Xia M, Xu X, editors. *Assay Guidance Manual* [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-2013 May 1 [updated 2016 Jul 1].
- 14) Pazhouhi M, Sariri R, Khazaei MR, Moradi MT, Khazaei M. Synergistic effect of temozolomide and thymoquinone on human glioblastoma multiforme cell line (U87MG). *J Cancer Res Ther* 2017; 1023-1028.
- 15) Khazaei M, Pazhouhi M. Temozolomide-mediated apoptotic death is improved by thymoquinone in U87MG cell line. *Cancer Invest* 2017; 35: 225-236.
- 16) Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol* 2005; 5: 12.
- 17) Pazhouhi M, Sariri R, Rabzia A, Khazaei M. Thymoquinone synergistically potentiates temozolomide cytotoxicity through the inhibition of autophagy in U87MG cell line. *Iran J Basic Med Sci* 2016; 19: 890-898.
- 18) Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors* 2003; 3: 276-284.
- 19) Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 2010; 70: 440-446.
- 20) Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 2009; 136: 823-837.
- 21) Huang HP, Chang YC, Wu CH, Hung CN, Wang CJ. Anthocyanin-rich mulberry extract inhibit the gastric cancer cell growth in vitro and xenograft mice by inducing signals of p38/p53 and c-jun. *Food Chem* 2011; 129: 1703-1709.
- 22) Shahneh FZ, Baradaran B, Majidi J, Babaloo Z. *Echinophora platyloba* DC (Apiaceae) crude extract induces apoptosis in human prostate adenocarcinoma cells (PC-3). *Biomed J* 2014; 37: 298-304.
- 23) Gali-Muhtasib H, Hmadi R, Kareh M, Tohme R, Darwiche N. Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. *Apoptosis* 2015; 20: 1531-1562.
- 24) Durak ZE, Buber S, Kocaoglu EH, Ozturk B. Aqueous extracts of celandine, red clover, flax seed and coriander inhibit adenosine deaminase enzyme activity in cancerous human gastric tissues. *Am J Food Sci Health* 2015; 1: 51-56.
- 25) Booth NL, Overk CR, Yao P, Burdette JE, Nikolic D, Chen SN, Bolton JL, van Breemen RB, Pauli GF, Farnsworth NR. The chemical and biologic profile of a red clover (*trifolium pratense* L.) phase ii clinical extract. *J Altern Complement Med* 2006; 12: 133-139.
- 26) Kaurinovic B, Popovic M, Vlasisavljevic S, Schwartzova H, Vojinovic-Miloradov M. Antioxidant profile of *trifolium pratense* L. *Molecules* 2012; 17: 11156-11172.
- 27) Amawi H, Ashby CR Jr, Tiwari AK. Cancer chemoprevention through dietary flavonoids: what's limiting? *Chin J Canc* 2017; 36: 50.
- 28) Mahajan P, Gnana Oli R, Jachak SM, Bharate SB, Chaudhuri B. Antioxidant and antiproliferative activity of indigocarpan, a pterocarpan from *Indigofera aspalathoides*. *J Pharm Pharmacol* 2016; 68: 1331-1339.
- 29) Thakur A, Singla R, Jaitak V. Coumarins as anticancer agents: a review on synthetic strategies, mechanism of action and SAR studies. *Eur J Med Chem* 2015; 101: 476-495.
- 30) Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 2000; 45: 528-537.
- 31) Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; 35: 495-516.
- 32) Tor YS, Yazan LS, Foo JB, Armania N, Cheah YK, Abdullah R, Imam MU, Ismail N, Ismail M. Induction of apoptosis through oxidative stress-related pathways in MCF-7, human breast cancer cells, by ethyl acetate extract of *Dillenia suffruticosa*. *BMC Complement Altern Med* 2014; 14: 55.
- 33) Maycotte P, Thorburn A. Autophagy and cancer therapy. *Cancer Biol Ther* 2011; 11: 127-137.
- 34) Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; 35: 495-516.
- 35) Floyd RA, Kotake Y, Towner RA, Guo WX, Nakae D, Konishi Y. Nitric oxide and cancer development. *J Toxicol Pathol* 2007; 20: 77-92.
- 36) Kim YM, Bombeck CA, Billiar TR. Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res* 1999; 84: 253-256.