Original Article

Colchicine of *Colchicum autumnale,* A Traditional Anti-Inflammatory Medicine, Induces Apoptosis by Activation of Apoptotic Genes and Proteins Expression in Human Breast (MCF-7) and Mouse Breast (4T1) Cell Lines

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Abstract

Objective: Breast cancer is one of the major causes of mortality among women. Due to many side effects of the existing chemotherapeutic agents, the research of anti-cancer drugs, including natural products, is still a big challenge. Here, we investigated the effects of colchicine on apoptosis of two breast cancer cell lines (human MCF-7 and mouse 4T1).

Materials and Methods: In this experimental study, we evaluated the apoptotic effects of colchicine on (MCF-7) and (4T1), as well as a human cancer-associated fibroblast cell line as a control group. Extraction and chromatographic techniques were applied to isolate colchicine from *Colchicum autumnale* L. To compare the isolated colchicine with pure standard colchicine, we used the H-NMR technique. The methyl thiazolyl tetrazolium (MTT) assay, quantitative reverse transcriptase-polymerase chain reaction, Western blotting and annexin V/PI staining were used to evaluate the apoptotic effects of the isolated and standard colchicine.

Results: Similar to standard colchicine, the isolated colchicine inhibited cell proliferation significantly in cancer cell lines. Colchine inhibited proliferation and induced apoptosis on a dose-dependent manner. The medicine modified the expression of genes-related to apoptosis by up-regulation of *P53 ,BAX, CASPASE-3, -9* and down-regulation of *BCL-2* gene, which led to an increase in the BAX/BCL-2 ratio.

Conclusion: We showed that isolated colchicine from *Colchicum autumnale* and pure standard colchicines modulate the expression levels of several genes and therefore exerting their anticancer effects on both human (MCF-7) and mouse (4T1) breast cancer cells. Based on these results, we suggest that colchicine can be a potential candidate for prevention and treatment of breast cancer.

Keywords: Apoptosis, Breast Cancer Cell, Colchicine, *Colchicum autumnale*, Toxicity

Introduction

As the most common cancer among women, breast cancer is a major public health problem around the world, in both developed and developing countries. Based on World Health Organization (WHO), over 502,000 women die annually from breast cancer and it also results in more than $7 billion medical costs per year, worldwide. Unfortunately, the mortality rate due to this type of cancer has dramatically increased in the past decade in Iran (1, 2). Recently, chemotherapy and radiotherapy (lonely or in combination) are the common treatments for breast and other types of cancers as well. But besides their therapeutic effects, they induce wide undesired effects on healthy cells as well (3).

Colchicine is a tropolane alkaloid, widely used for its anti-gout and anti-inflammatory activities (4-6). Colchicine by binding to the tubulins and interfering with their polymerization, results in disruption of mitosis. It also leads to the inhibition of leukocytes and migration of other inflammatory cells (7, 8). Besides, colchicine increases the cellular free tubulins to limit the mitochondrial metabolism in cancer cells by blocking voltage-dependent anion channels of the mitochondrial membrane (9). Other studies have also reported the antiproliferative and anticarcinogenic properties of colchicine in different cell lines and animal models. Additionally, recent studies in this area have shown that colchicine as an anticancer agent, has a high potential to induce apoptosis in cancer cells. This is evidenced by its growth inhibitory effect on certain types of tumor cell lines both *in vitro* and *in vivo*, such as gastric cells (10, 11), hepatocellular carcinoma (12), liver cells (13), and cholangiocarcinoma (14). Colchicine at high doses is extremely toxic, which limits its use in therapy on human cells (10). Apoptosis is a highly regulated process that begins by activation of various molecules in this pathway.

Therefore, the activations of these molecules in the apoptosis pathway offer promising approaches towards cancer therapy. However, the exact cytotoxic mechanism of colchicine is still unknown due to the certain anti- and pro-apoptotic signaling pathways in different cell types. Since colchicine is extremely toxic at high doses (15), the amount of it that may be used on cells is limited, and thus it is a big challenge to determine the mechanism behind its apoptotic effects on breast cancer cells.

In this study, we evaluated the cytotoxic and growth inhibitory effects of isolated colchicine from *C. autumnale* L., and investigated its effects on apoptotic genes and proteins in human (MCF-7) and mouse (4T1) breast cancer cell lines.

Materials and Methods

Extraction and purification of colchicine from *Colchicum autumnale L. corms*

This report is on an experimental study, in which the corms of *Colchicum* were collected from Kermanshah, Iran, between October and November 2014. Corms was further identified and confirmed by the Department of Agricultural College (Voucher number: 2770, deposited in Herbarium of Razi University, director: Dr. S M. Maassoumi). Extraction was performed according to Alali et al. (16) with slight modifications. Brieftly, the bulbs were crushed and dried out in the dark and then powdered with a laboratory grinder. One weight of the powder was extracted two times with 50 volumes of ether while shaking for 2 hours, followed by filteration. The solid residues were air-dried and extracted with dichloromethane (DCM) at room temperature for 30 minutes by shaking. Then, 10% ammonia solution was added to the mixture with intense shaking for 10 minutes and the mixture was left undisturbed for the next 60 minutes. Right after the break, 250 ml of DCM was added and the mixture was immediately filtered. The residue was washed twice with 250 ml of DCM and then pooled with the filtrate. The solution was allowed to evaporate to dry.

Column chromatography was performed on silica gel (Merck, Darmstadt, Germany; 63-200 µm particle size) that was already slurry-packed with DCM. The dried extract from the previous step dissolved in DCM and was applied to the column. The column was eluted by using chloroform-methanol solution (5:95). The eluted fractions were evaluated through thin layer chromatography (TLC) and then visualized by ultraviolet (UV) at 254 nm. In the eluted fractions colchicines were identified by TLC and classified into five groups based on the band intensity. Each of the five groups was evaporated to dryness. In the next step, the content of each group was evaluated by TLC and compared to the pure standard colchicine powder from Alexis (Nottingham, East Midlands, UK). Two fractions that showed the highest similarity to the standard control colchicine were further tested for their effectiveness.

For quantitative analysis of the isolated colchicine, we used the UV spectroscopy method (Bioaquarius, Cecile,

UK) with the standard control colchicine as the reference. Moreover, a known amount of colchicine was dissolved in methanol and checked for its λref value with UV spectroscopy. In order to run Fourier-transform infrared (FTIR) spectroscopy (PerkinElmer- M-15 model), the sample was prepared according to the protocol and the results were analyzed by SHIMADZU (IR Prestige-21 model).

Finally, the H-NMR method was used to determine the similarity of the isolated colchicine from *C. autumnale* L to the standard control colchicine. The H-NMR spectra in the experiment were recorded on a 500 MHz (Bruker, Germany) instrument, with chloroform ($CDCl_3$) as the solvent.

Cultivation of cell lines

Human (MCF-7) and mouse (4T1) breast cancer cell lines were obtained from Pasteur Institute of Iran and human fibroblast cells were isolated from normal-healthy skin used as the control [all experimental procedures were carried out in accordance with the Ethical Principles and the National Norms and Standards for Conducting Medical Research in Iran (IR.KUMS.REC.1397.941)]. Informed consent was obtained from all individual participants included in the study. The human and mouse breast cancer cells (MCF-7, 4T1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Incubation was carried out at 37°C with 5% CO_2.

Cytotoxicity assay

MCF-7, 4T1, and fibroblast cells were cultured in 96-well plates in DMEM containing 10% fetal calf serum (FCS), at 37ºC and 5% CO_2 and were allowed to reach 70-80% confluency. Culture medium after 24 to 48 hours (by cell type) was replaced with 300 µl fresh medium containing 5% FCS. The wells were treated with different concentrations of isolated and standard colchicine and some wells were assigned as controls (untreated). After 24 hours, cells were detached from the plate using trypsin-EDTA and resuspended in a 100 µl of culture medium after centrifugation. The suspension was well dispersed and then the viability of the cells was evaluated after adding trypan blue (0.4% in PBS) to the test and control wells. The following formula was applied to calculate the viability at different time intervals:

Viability% = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

Growth inhibition using MTT assay

Human (MCF-7) and mouse (4T1) breast cancer cell lines and normal human fibroblasts were seeded (5000 cells/wells) in 96-well plates and incubated in a serum-containing medium, as mentioned above, for 48 hours. Then, cells were treated with various concentrations of purified and isolated colchicine (1-400 ng/ml)

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while control wells remained untreated. The cells were incubated for an additional 48 hours, thereafter 200 μl of MTT solution (5 mg/ml, filtered) was added to each well and the cells were incubated for another 3-4 hours. Dimethyl sulfoxide (DMSO) was added (150 μl) to each well after removal of the medium and the absorbance was measured in a microplate reader at 570 nm.

Quantitative analysis of reverse transcriptase-polymerase chain reaction

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) was used to synthesize cDNA. To prevent the formation of primer-dimers and non-specific products during real-time polymerase chain reaction (PCR), melt curve analysis and PCR were also done. Gene expression was assessed by real-time quantitative PCR using the Real-Time PCR system (Rotor-Gene 6000 Cycler, Qiagen, Germany) and the Power SYBR Green PCR master mix. Primers were selected for each cell line and for the target genes *BAX, BCL-2, P53* and *β-ACTIN* (MCF-7 cell line), *Bax, Bcl-2, p53* and *β-Actin* (4T1 cell line) based on the nucleotide sequences downloaded from the Pre Primer software. Furthermore, the specificity of the primers was checked by the National Center for Biotechnology Information data bank. To ensure the absence of singly nucleotide polymorphism (SNP), these primers were reviewed on the site of the NCBI checker.

The real-time PCR for relative quantification of the target genes was performed in a total volume of 20 μl comprised of 10 μl SYBR® Premix Ex TaqTM master mix (Takara, Japan), 0.5 μl of each forward and reverse primer, 0.08 μl diluted ROX, and 2 μl DNA template, under following conditions: an initial denaturation of templates at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds. The reaction was ended after the final extension at 75°C for 15 seconds. A melt curve analysis was performed to assess the formation of primer-dimer or non-specific products during the amplification. Accordingly, at the last step of the amplification, the reaction was continued by heating at 95°C for 15 seconds, a temperature gradient of 60-95°C for 1 minute, and 95°C for 15 seconds. The relative expression genes (both cell line) in the treated and control mice was calculated by using the formula $2^{-\Delta\Delta ct}$ presented below.

Western blot

To detect changes in apoptosis-related proteins (p53, Bcl-2, Bax, caspase-3 and caspase-9), MCF-7 and 4T1 cells (1×10^7 cells) were seeded in $10cm^2$ dishes and treated with 0.5 and 1μg/ml of isolated colchicine and 0.2 and 0.4 μg/ml of pure standard colchicine. After 24 hours, the cells were harvested and lysed using a lysis buffer (50 mM Tris-HCL, pH=8, 8 M urea, 2 M thiourea and 0.1 mM PMSF). The protein concentration of the cell lysates

was quantified according to the method of Bradford with BSA as the standard. Total protein (35 μg) was run onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 60 minutes. Then, the protein bands were transferred to a polyvinylidene difluoride membrane (PVDF, Roche,Germany). The membranes were blocked with PBS containing Tween 0.5% and BSA 2% for 20 minutes and incubated overnight at 4°C with the following primary antibodies (Santa Cruze): anti-p53 (1:300), anti-Bcl-2 (1:300), anti-Bax (1:300), anti-b-actin (1:1000) anti-caspase-3 (1:500), and anti-caspase-9 (1:500). After washing the membrane in PBS containing Tween 0.05% (PBS-T) four times, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody for detection. After washing in PBS-T (four times), the reactive bands were visualized using 3,3´- diaminobenzidine (DAB) and H_2O_2 substrate solution. Finally, the reactive bands were analyzed by Image J software (LOCI, U.S). The relative fold-difference was calculated by the integration of the normalized to the intensity of the ß-actin band.

Annexin-V/PI staining assay

The PI and annexin V-FITC double-binding assay was applied to detect apoptotic cells by annexin V-FITC/PI kit. Cells were seeded in 6-well plates containing complete medium for 24 hours and then in a 24-hours treatment, MCF7 and 4T1 cells were exposed to the isolated (0.5 and 1 μg/ml) and control (0.2 and 0.4 μg/ml) colchicine. As stated in the kit manual, we harvested the cells, rinesed twice with PBS and gently resuspended in annexin V binding buffer and incubated with annexin V-FITC/ PI in the dark for 10 minutes. Ultimettly, the cells were evaluated by fluorescence microscopy (Ts100, Nikon, Tokyo, Japan) with at least 1×10^6 cells per sample. The investigation was repeated 3 times for each group.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 for Windows (SPSS, Chicago, IL, USA), where the data is reported as means ± standard error (SE). Differences between the isolated colchicine treated, standard colchicine treated and control groups (untreated) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Significance levels were determined by the Student t test. P<0.05 was considered statistically significant.

Results

Physicochemical characters of colchicine from *C. autumnale*

The isolated colchicine from *C. autumnale* L. was compared to standard colchicine (Nottingham, East Midlands, UK) using Fourier Transform Infrared Spectroscopy (FTIR), Proton Nuclear magnetic resonance spectroscopy (H-NMR) and UV spectroscopy to determie the purity and similarity of the isolated colchicines.

The results of FTIR and H-NMR confirmed the same molecular structure in both types of colchicines. The results of the UV scan determined a λ max value of 243.5 and 350 nm for pure standard colchicine; completely similar to what is stated in previous studies. Isolated colchicine also had a maximum absorption of approximately 200-400 nm, which is about the same as the standard colchicine. Therefore, in this study, isolated colchicine from *C. autumnale* L. has been used as an anti-cancer agent on MCF-7, 4T1 cells.

Cytotoxic effects of colchicine on MCF-7 and 4T1 cells

Trypan Blue exclusion test was applied for investigation of the cytotoxic effects of colchicine on human and mouse breast cancer cells, as well as on fibroblasts. The results from MCF-7 cells exposed to various concentrations (0.002-8 µg/ml) of the standard and isolated colchicine during a 24 hours period are shown in Figure 1A. The cytotoxic effect was significant, indicated by the uptake of trypan blue dye, at the concentrations of greater than or equal to 0.1 µg/ml and 0.5 µg/ml for isolated and standard colchicine, respectively ($P<0.05$). Additionally, 50% Cytotoxicity Concentrations (CC50) of the isolated and standard colchicines on MCF-7 cells were 2 µg/ml and 1 µg/ml, respectively (Fig.1A). For further investigations, concentrations close to the CC_{50} values (0.5 and 1 µg/ml) were selected.

The results from treating the 4T1 cell line with various concentrations (1-800 µg/ml) of standard and isolated colchicine for a 24 hours period are shown in Figure 1B. Cytotoxicity of both compounds on mouse breast cancer cells was significant at concentrations ≥ 10 µg/ml ($P<0.05$). Furthermore, the CC_{50} for both compounds was similar (300 µg/ml, Fig.1B). We observed significant cytotoxic activity on human fibroblast cells when using concentrations above 0.1 µg/ml for isolated and 0.4 µg/ml for standard colchicine ($P<0.05$). The CC_{50} of colchicine for human fibroblast was 6 µg/ml (Fig.1C).

The growth inhibitory effect of colchicine on MCF-7 and 4T1 cells

Growth inhibitory activity of colchicine on human and mouse breast cancer cells and human fibroblasts was evaluated after treating the cells with either form of colchicine for 48 hours. Thereafter, the viability of the cells was estimated using MTT assay. The MCF-7, 4T1 and fibroblast cells that were exposed to standard and isolated colchicine showed a significant decrease in cell growth in a dose-dependent manner. The inhibitory effects of the two colchicines on MCF-7 cells was remarkable at concentrations ≥ 8 ng/ml ($P<0.001$). Finally, 50% inhibitory concentration (IC_{50}) of both isolated and standard colchicines were 8 ng/ml and 4 ng/ml, respectively (Fig.2A).

In the case of 4T1 cells, the effective inhibitory concentrations of the isolated and standard colchicine started from ≥ 20 µg/ml and ≥ 100 µg/ml, respectively ($P<0.05$). But the IC_{50} value for both colchicines on 4T1 cells was 200 µg/ml (Fig.2B).

The significant growth inhibitory effect of the isolated and standard colchicines on fibroblast cells started from 0.4 µg/ml ($P<0.001$). The IC_{50} of both colchicines on fibroblast cells was 2 µg/ml (Fig.2C).
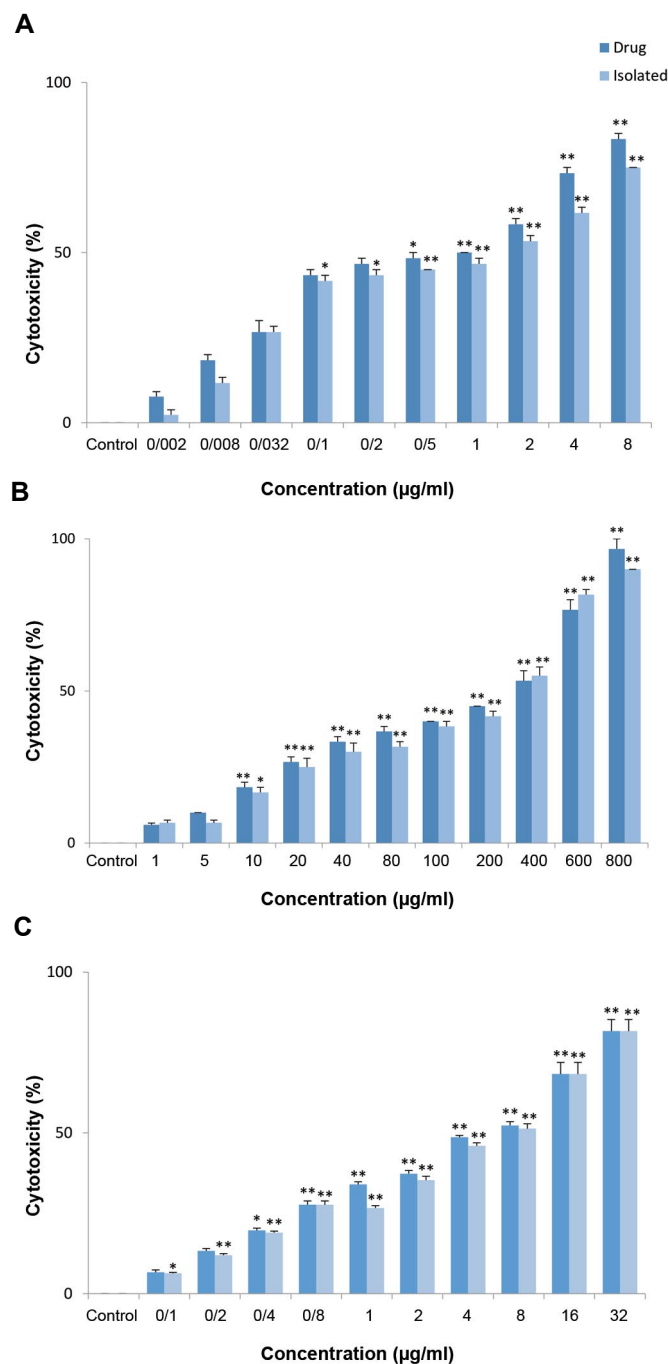

Fig.1: Cytotoxic effects of different concentrations of standard (drug) and isolated colchicine after 24-hours of treatment. **A.** On MCF-7 cells, **B.** On 4T1 cells, and **C.** On fibroblast cells. Data are given as mean ± SE for each point of three separate experiments, *; P<0.05, **; P<0.001 vs. control.
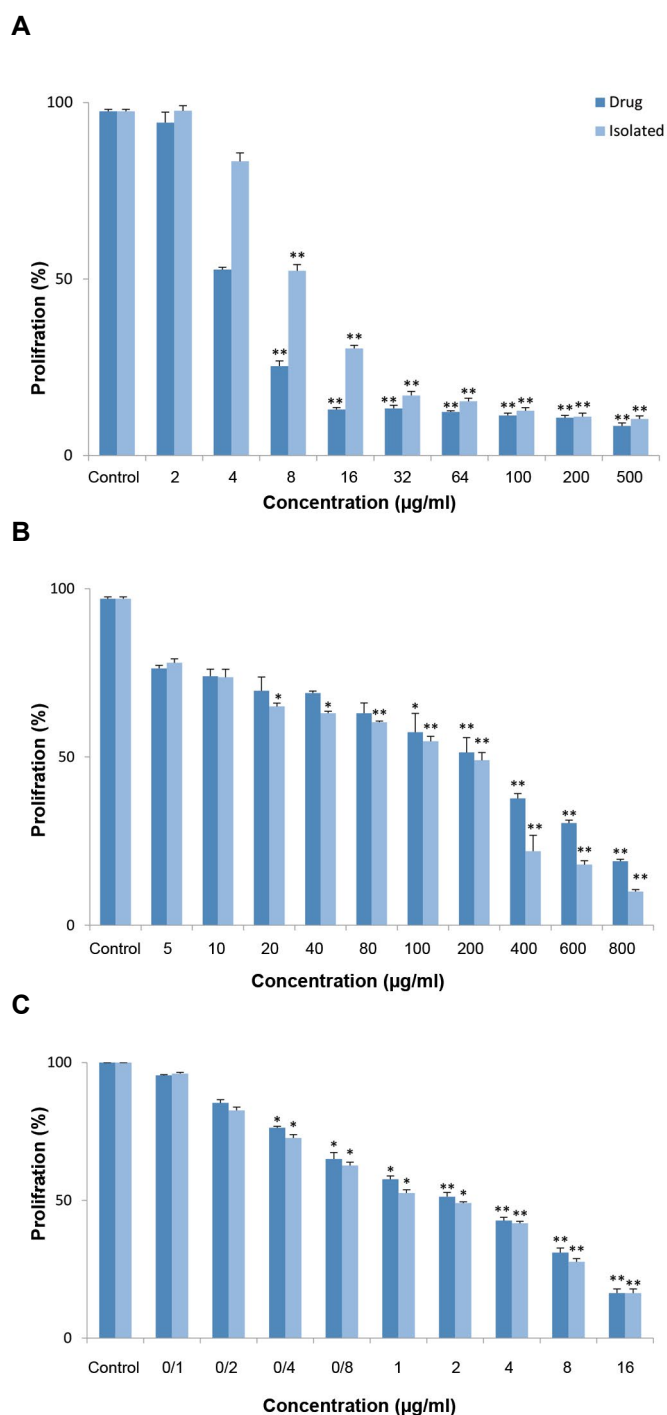
A

B

C

Fig.2: The growth inhibitory effect of different concentrations of isolated and standard colchicine (drug) after 48 hours. **A.** On MCF-7 cells, **B.** 4T1 cells, and **C.** Fibroblast cells. Data are given as mean ± SE for each point of three separate experiments, *; P<0.05 and **; P<0.001 vs. control.

The effects of colchicine on the expression of *P53*, *BAX* and *BCL-2* genes

The effects of isolated and standard colchicine on expression of the three mentioned genes *P53, BAX* and *BCL-2* in MCF-7 cells was examined by quantitative RT-PCR. Both compounds induced an up-regulation of *P53* gene (2.75-folds and 1.22 folds at 0.5 µg/ml,

and 1.68 folds and 2.47 folds at 1 µg/ml, respectively) and *BAX* gene (5-folds and 2.07-folds at 0.5 µg/ml, 1.61-folds and 2-folds at 1 µg/ml, respectively). However, both colchicines down-regulated *BCL-2* gene (0.74-fold and 0.59-fold at 0.5 µg/ml, and 0.7fold and 0.53-fold at 1 µg/ml, respectively). Additionally, significantly different gene expression levels were observed between the two cell types when using equal concentrations of the standard and the isolated colchicine (*P<0.01, **P<0.05, and ***P<0.001), This significant difference was also observed when using different concentrations (0.5 and 1 µg/ml) of the two colchicines (Fig.3A, *P<0.01, **P<0.05, and ***P<0.001).

Investigating the expression levels of *P53*, *Bax* and *Bcl-2* genes in 4T1 cells treated with isolated and standard colchicine at 200-400 µg/ml concentrations demonstrated an up-regulation in the expression of *P53* gene (1.07-folds and 1.29-folds at 200 µg/ml, 2.73-folds and 2.04-folds at 400 µg/ml of the isolated and standard colchicine, respectively) and *Bax* gene (6.27-folds and 4.43-folds at 200 µg/ml, 1.72-folds and 1.44-folds at 400 µg/ml of both compounds, respectively) but a down-regulation of *Bcl-2* gene (0.21-fold and 0.43-fold at 200-µg/ml, 0.61-fold and 0.18-fold at 400 µg/ml of the isolated- and standard-colchicine, respectively). Similar to MCF-7 cells, significantly different effects on gene expression levels in the 4T1 cells were observed using equal concentration of the isolated and the standard colchicine (*P<0.01, **P<0.05, and ***P<0.001). Significant differences in the effects of our treatments on gene expression levels at two different concentrations (200 and 400 µg/ml) of standard and the isolated colchicine was found as well (Fig.3B, *P<0.01, **P<0.05, and ***P<0.001).

BCL-2 superfamily plays an important role in cell apoptosis. Two members of the *BCL-2* family are anti-apoptotic *BCL-2* and pro-apoptotic *BAX*. Isolated colchicine increased the *BAX/BCL-2* ratio (as an index of apoptosis) in MCF-7 at the concentrations of 0.5 and 1 µg/ml and in 4T1 cells at 200 and 400 µg/ml. Isolated colchicine raised the *BAX/BCL-2* ratio up to 4.5 fold and 1.3 fold at the concentrations of 0.5 and 1 µg/ml, respectively in the MCF-7 cells compared to the control group. Standard colchicine also lead to the same outcome but in different amounts (1.6 fold and 1 fold at 0.5 and 1 µg/ml, respectively). In the 4T1 cells, standard colchicine increased this ratio up to 2.9 fold and 2.8 fold at concentrations of 200 and 400 µg/ml, respectively. Besides that, isolated colchicine caused a more remarkable increase in *BAX/BCL-2* ratio, which was 10.8 fold and 8 fold at the concentrations of 200 and 400 µg/ml, respectively (Fig.3C).
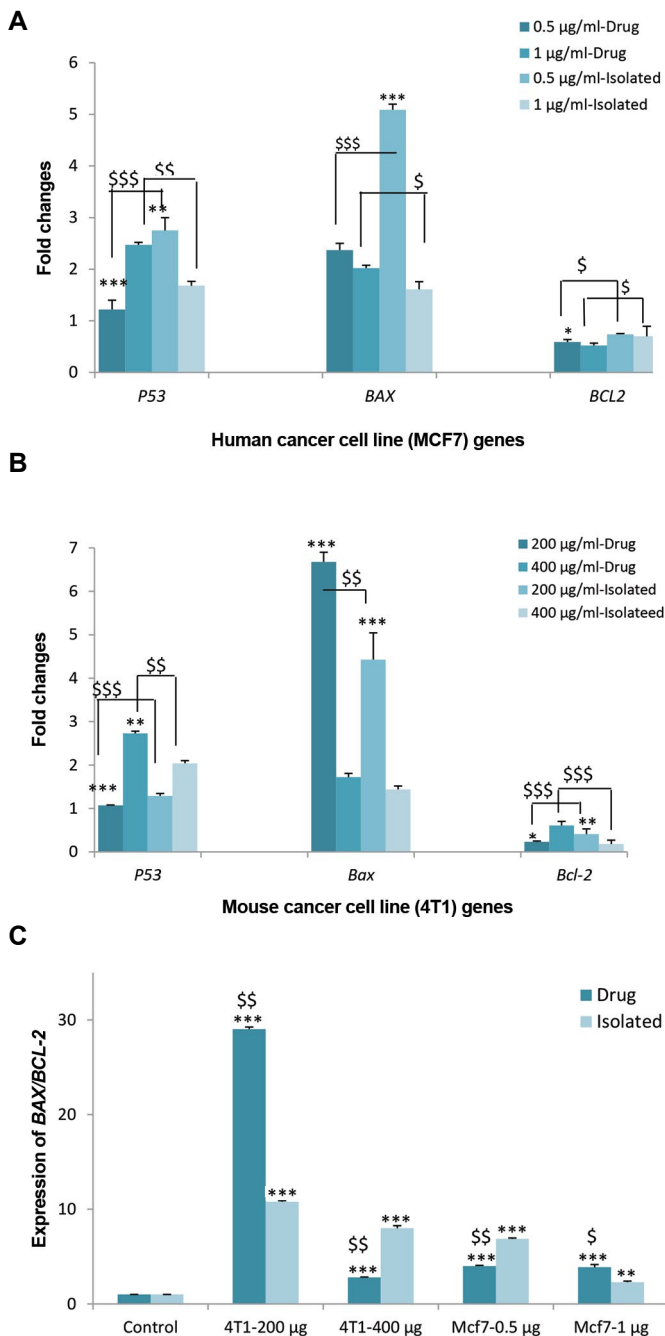
Fig.3: mRNA expression of pro-apoptotic and anti-apoptotic genes. **A.** *P53, BAX, BCL-2* in MCF-7 cells, **B.** *p53, Bax, Bcl-2* in 4T1 cells. The expression of pro-apoptotic and anti-apoptotic genes in both cell lines was determined by measuring mRNA levels using real-time polymerase chain reaction (PCR). Data are given as mean ± SE for each point of three separate experiments, *; $P<0.01$, **; $P<0.05$, ***; $P<0.001$, between equal concentrations of colchicine and $; $P<0.01$, $$; $P<0.05$, $$$; $P<0.001$, between different concentrations of colchicine. **C.** Colchicine effects on the expression of *BAX/BCL-2* in MCF-7 and 4T1 cells. Data are given as mean ± SE for each point of three separate experiments. *; $P<0.01$, **; $P<0.05$, ***; $P<0.001$ vs. control and $; $P<0.01$, $$; $P<0.05$, $$$; $P<0.001$ vs. equal concentrations of isolated and standard colchicine (drug).

The effect of colchicine on the level of apoptosis-related proteins

In this experiment, the effect of colchicine-induced apoptosis on MCF-7 and 4T1 cells was investigated, the constitutive expression of typical apoptosis-related proteins of P53, Bax, Bcl-2, Caspas-3, Caspase-9 and β-Actin (as the reference protein) by Western blot method. The results showed that after 24 hours of treatment, the levels of pro-apoptotic proteins (P53 and Bax) increased, while the expression of anti-apoptotic proteins of the Bcl-2 family decreased (Fig.4A).

Compared to the controls (untreated group), treating MCF-7 cells with 0.5 µg/ml of standard colchicine led to an 82% decrease in pro-apoptotic protein Bax level (Fig.4B). The isolated colchicine also induced the same effect on Bax level (85%) in the these human cells. However, at higher concentrations of both standard and isolated colchicine (1 µg/ml), 27% and 5% increases were seen in Bax level, respectively. The expression level of the anti-apoptotic protein Bcl-2 in the treated cells with 0.5 µg/ml of the standard colchicine significantly decreased (94%) but isolated colchicine at the same concentration increased the Bcl-2 level by 2.7%. On the other hand, 1µg/ml of the standard colchicines caused a 13% decrease in Bcl-2 level, but the isolated colchicine induced a 1.5% increase in Bcl-2 expression level.

The level of pro-apoptotic protein, Bax, in the case of 4T1 cells showed an 87% decrease and 83% increase after exposure to 200 µg/ml of the standard and the isolated colchicine, respectively (Fig.4C). But at a higher concentration (400 µg/ml), the standard and isolated colchicine had opposite effects (64% increase and 7% decrease, respectively). The expression level of the anti-apoptotic protein of Bcl-2 in 4T1 cells treated with 200 µg/ml of the standard and the isolated colchicine was decreased by 55% and 41%, respectively. This is while at 400 µg/ml, the standard and isolated colchicines induced 42% and 44% decrease in Bcl-2 expression level, respectively.

The effect of colchicine on Caspase-3, -9 activities in MCF-7 and 4T1 cells

Based on the results, which are presented in Figures 4B, C, the expression levels of Caspase-3, -9 protein decreased compared to the controls. By considering that our used antibodies detected the pre-caspases, reduction in the pre-caspases level can be due to their turnover into active caspases in the apoptosis pathway.

Apoptotic effect of colchicine on MCF-7 and 4T1 cells

100 treated and 100 untreated cells were counted from MCF-7 and 4T1 cell lines under the fluorescence microscope after Annexin-V/PI staining to determine the early and late apoptosis (Figs.5, 6). After 24-hours of treatment with the isolated and standard colchicine, the early apoptosis percentage of the treated MCF-7 cells at the dose of 0.5 µg/ml were 20% and 24% and at the dose of 1 µg/ml were 14% and 18%, respectively. These results showed a significant apoptotic effect compared to the controls (*$P<0.05$ and **$P<0.001$).

A significant difference was also observed between the late and early apoptosis at the same concentration of the isolated and standard colchicine ($^{\$}$P<0.05 and $^{\$\$}$P<0.001, Fig.5F).

In the case of 4T1 cells treated with the isolated and standard colchicines, the early apoptosis percentage was determined as 12% and 30% at 200 µg/ml and 6% and 8% at 400 µg/ml, respectively (Fig.6F). That means a significant apoptotic effect of both colchicines (*P<0.05 and **P<0.001). Additionally, a significant difference was also observed between the late and early apoptosis with equal concentrations of the isolated and standard colchicines (*P<0.05 and **P<0.001, Fig.6F).
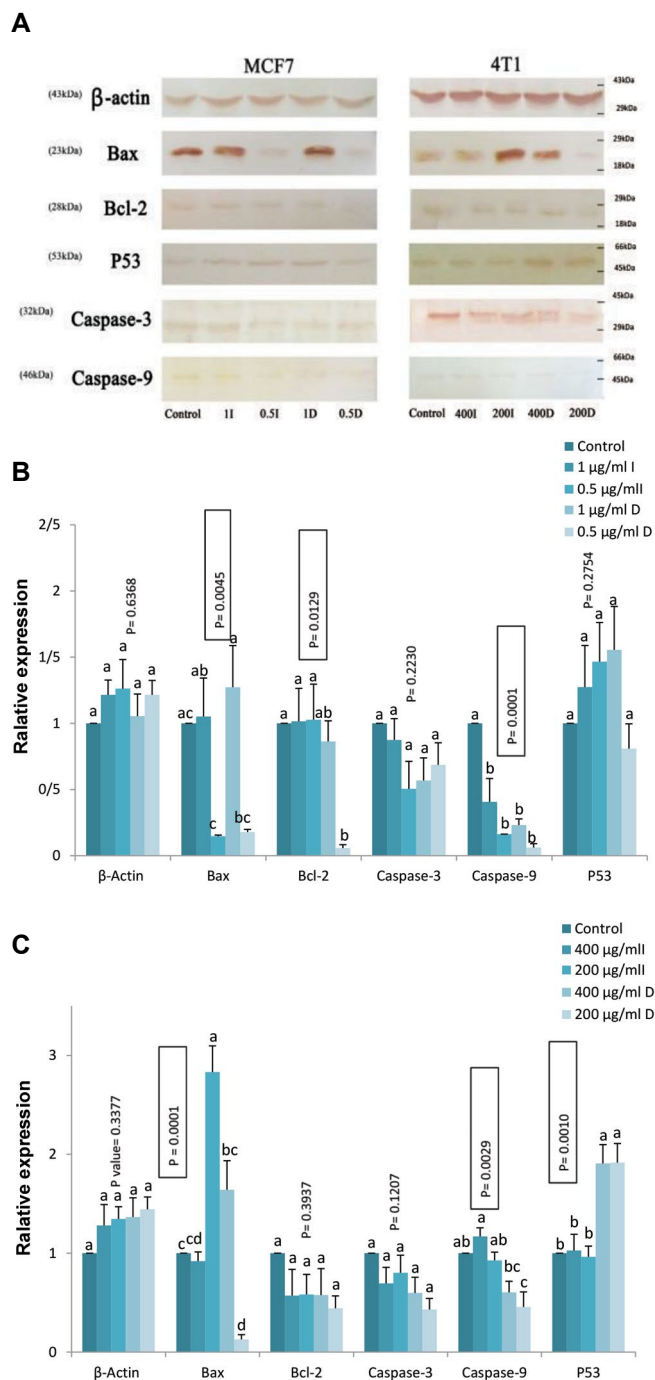

Fig.4: Expression of pro-apoptotic and anti-apoptotic proteins and densitometric analysis. **A.** The effects of colchicine on the expression of pro-apoptotic and anti-apoptotic proteins in MCF-7 and 4T1 cells. Cells were exposed to the isolated and standard colchicine (drug) at 0.5 and 1 µg/ml and 200 and 400 µg/ml, respectively for 24 hours. The protein expression of P53, Bax, Bcl-2, caspase-3 and -9 was determined by Western blotting against controls, **B**. Densitometricy analysis of P53, Bax, Bcl-2, Caspase-3 and -9 proteins in MCF-7 cell line, and **C**. Densitometricy analysis in 4T1 cell line. Data are given as mean ± SE for each point of three separate experiments. Different letters indicate a significant difference (P<0.05) and the same characters indicate a non-significant difference between the treatments (P>0.05). I; Isolated and D; Drug.
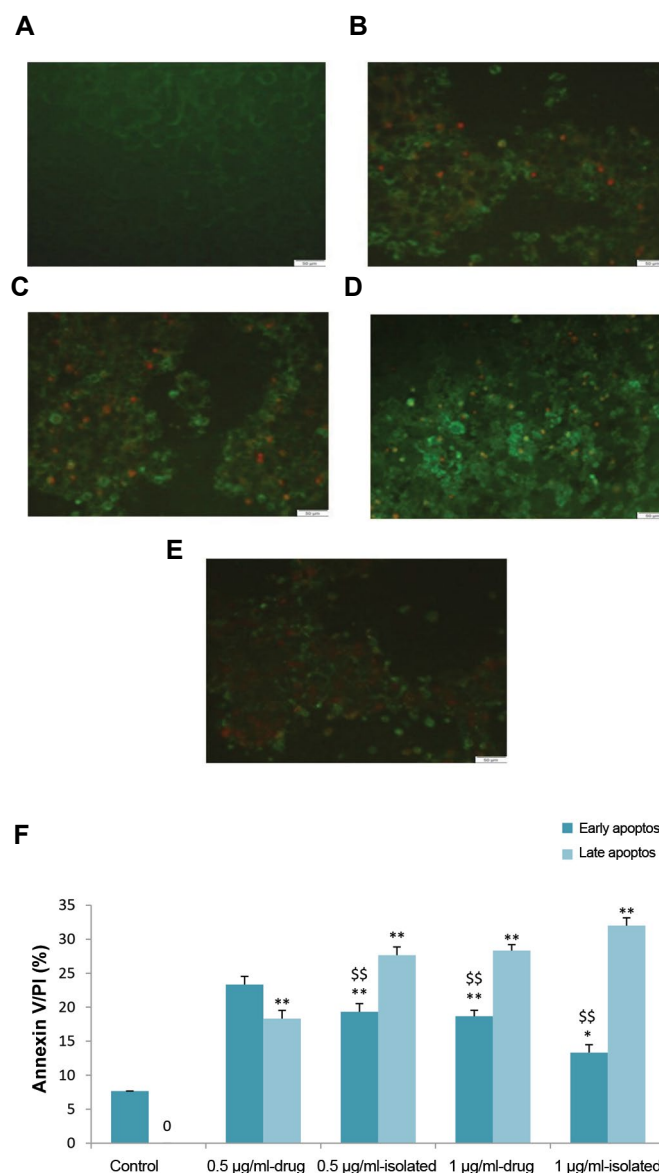
Fig.5: Results of Fluorescence Microscope image and early and late apoptos of MCF-7 cells. **A.** Untreated cells as the control group, **B.** Treated cells with 0.5 µg/ml of standard colchicine (drug), **C.** Treated cells with 1 µg/ml of standard colchicine (drug), **D.** Treated cells with 0.5 µg/ml of isolated colchicine, **E.** Treated cells with 1 µg/ml of isolated colchicine (scale bar: 50 µm), and **F.** The results of early and late apoptosis in MCF-7 cells determined by Annexin V-PI method. **; P<0.001 vs. control, and $^{\$\$}$; P<0.001 vs. equal concentrations of isolated and standard colchicine (drug).

A

B

C

D

E

F
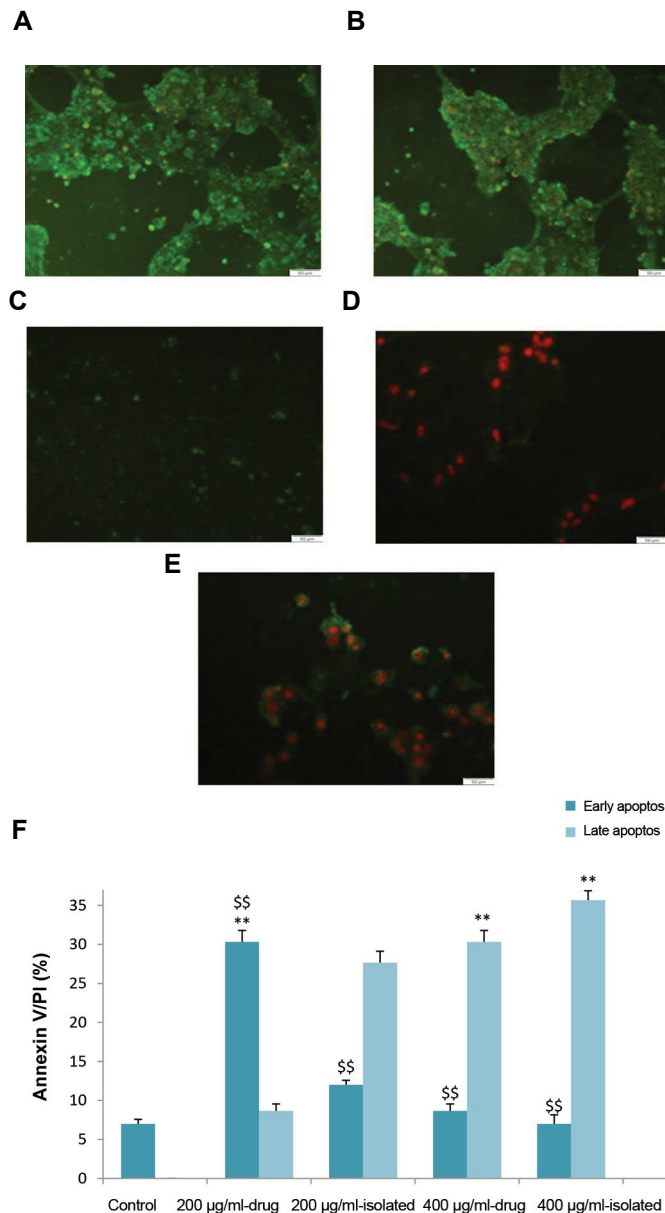

Early apoptos
Late apoptos

Fig.6: Results of Fluorescence Microscope image and early and late apoptos of 4T1 cells. **A.** Untreated cells as control group, **B.** Treated cells with 200 μg/ml of standard colchicine, **C.** Treated cells with 400 μg/ml of standard colchicine (drug), **D.** Treated cells with 200 μg/ml of isolated colchicine, **E.** Treated cells with 400 μg/ml of standard colchicine (drug) (scale bar: 50 μm), and **F.** Results of early and late apoptosis in 4T1 cells determined by Annexin V-PI method. **; $P<0.001$ vs. control and $$; $P<0.001$ vs. same concentration of isolated and standard colchicine (drug).

Discussion

Herein, for the first time, we try to uncover some possible effects of isolated colchicine of *Colchicum autumnale* on MCF-7 and 4T1 cell cycle, growth and apoptosis. The isolated colchicine, which was (compared to standard colchicine) induced cytotoxic effects on MCF-7 and 4T1 cells in a dose-dependent manner. Our results revealed that low concentrations of colchicine (1-2 μg/ml) had a significant cytotoxic effect on the MCF-7 cell line compared to healthy human fibroblast cells (6 μg/ml). In contrast, the cytotoxic effect of colchicine on 4T1 cell line was observed at a concentration of 300 μg/ml (vs. 6 μg/

ml on fibroblasts). Purified and standard colchicines both appear to reduce the growth of cancer cells at much lower concentrations (4-8 ng/ml) compared to the cytotoxic concentrations. This suggest that at low concentration, colchicine has an antiproliferative effect on these cancer cells, while at high concentrations it turns out to be cytotoxic. This is a very crucial factor to consider in chemotherapy planning when choosing a suitable anti-cancer agent. However, these *in vitro* results need to be confirmed by *in vivo* investigation.

The cytotoxic and growth inhibition effects of colochicine on mouse breast cancer cells, however, were observed at 300 and 200 μg/ml, respectively. Previous studies have revealed that colchicine at the concentrations of 2 ng/ml, 4 ng/ml, 6 ng/ml and 0.04-4 μg/ml, has a significant anti-proliferative effect on cholangiocarcinoma, hepatocellular carcinoma (HCC), neuronal cells, gastric cancer cells and healthy liver cells (L-02), which can confirm our results (10-14). These results indicated that various cell types have different susceptibility and sensitivity to colchicine toxicity. Therefore, we believe that this remarkable concentration difference of colchicine to induces an anti-proliferative effect between these two cell lines can be possibly due to mutations in *p53* gene or other unknown mutations in the mouse breast cancer cells (4T1).

The cell cycle could be an appropriate target for anti-cancer drugs to induce apoptosis (17-19). Colchicine is a natural alkaloid that binds to tubulin, inhibits the formation of microtubules in mitotic spindles and therefore prevents cell division to finally be recognized as a well-known anti-cancer agent (5, 7, 20-22). Here, we have shown that both isolated and standard colchicine lead to different expression levels of several genes that have anti-cancer effects on human (MCF-7) and mouse (4T1) breast cancer cells.

Apoptosis, as a gene-controlled process, can be mediated through two pathways: mitochondrial (intrinsic) pathway or death receptor (extrinsic) pathway (23). More than 100 different genes have been identified to be involved in cell survival. It is revealed that two families of caspases and the *BCL-2* gene directly involved in apoptosis (24). The intrinsic pathway is initiated due to the release of mitochondrial proteins, for instance cytochrome c, which further binds to Apaf-1 and converts procaspase-9 to active caspase-9, leading to activation of caspase-3 and finally inducing apoptosis (25-27). It is well known that the balance between replication and apoptosis is interrupted in cancer cells, therefore apoptosis plays the key role in inhibition of their growth and proliferation. Accordingly, there is a reverse relationship between expression of the apoptosis-related genes and cancerous process.

In this study, the expression levels of apoptosis-related proteins including P53, Bax, Bcl-2, caspase-3 and caspase-9 activities were investigated to help us in understanding the molecular mechanisms of colchicine's anticancer and apoptosis-inducing effects. The results

indicated a dose-dependent up-regulation of P53 and Bax proteins, down-regulation of Bcl-2 and activation of caspase-3 and -9 as well. These results are consistent with the findings in other types of malignancies (28-31).

Several studies have demonstrated that apoptosis is mainly regulated by the Bcl-2 family members; either through activation of Bax or inhibition Bcl-2 (32). As it is revealed, during apoptosis the levels of Bcl-2 family proteins are significantly reduced while, the level of Bax is increased remarkabley (33). Our results indicated that colchicine-induced apoptosis relates to an augmented level of Bax and down-regulated Bcl-2, which finally lead to apoptosis of human and mouse breast cancer cells. In a study by Gundamaraju et al. (34), the effect of colchicine on the expression levels of *BAX* and *BCL-2* genes was evaluated in some mammalian cells. Their results showed a significant increase in the *BAX* gene expression level, while the *BCL-2* gene expression was unaffected. In our study, the level of *BAX* gene expression increased significantly, while in some cases the expression level of the *BCL-2* gene was not affected. The balance between *BCL-2* (anti-apoptotic) and *BAX* (pro-apoptotic) is the most critical parameter determining a cell's fate in response to extracellular stimuli.

This clearly suggests that increasing *BAX/BCL-2* ratio might be the means by which colchicine induces apoptosis. In many studies the apoptotic Mechanism of action of colchicine of colchicine has been assessed on neurons and liver cancer cells, but its exact mechanism of action, especially in breast cancer, is still unclear. In this study, the apoptotic features of colchicine are very similar to the results from other studies. These data indicate an increased expression level of Bax and p53 proteins, decreased expression of Bcl-2, and activated caspases-3 and -9. Therefore, based on the evidence and despite the lack of examination on the release of cytochrome c from mitochondria, it can be suggested that colchicine induces apoptosis in MCF-7 and 4T1 cells through the mitochondrial pathway (intrinsic pathway).

There are many questions yet to be answered that will require investigations beyond evaluating gene expressions. It has been demonstrated that protein production level is not quite in proportion to its mRNA level. Likewise, mRNA level is not often an exact indicator of protein expression, since they may contain multiple copies of one or a certain number of proteins (35, 36). In this regard, our results also showed that in our studied cell lines, there is no definite relationship between mRNA level and protein production of *P53, BCL-2* and *BAX* genes. However, the analysis of proteins involved in apoptosis clearly showed that both types of colchicine induce apoptosis (through increasing Bax and P53 and decreasing Bcl2 expressions) and interestingly, isolated colchicine was found as effective as the standard one.

Conclusions

In summary, we isolated colchicine from *C. autumnale*
L. with physicochemical and biological characteristics similar to standard colchicine. We showed that the apoptotic effects of the isolated colchicine from *C. autumnale* L. were as strong as the standard colchicine or even better in some cases, due to the tropolone alkaloids compounds. Further investigation is required to evaluate this combination for therapy. Moreover, appropriate *in vivo* (animal cancer models and then human) studies are needed in order to uncover additional possible colchicine's mechanisms of action.

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Authors' Contributions

A.M.; Conceived the first idea of the project, planned the experiments, and performed the final revision. A.M., Sh.I.; Supervised the project. E.A.F.; Acquisition of the experimental data and written the first draft of the manuscript. A.M., Y.Sh., Sh.I.; Performed the analysis and interpretation of the data. All authors read and approved the final manuscript.

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