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Original article

Cellular Behavior of *Colchicum troodi* Treated Primary and Metastatic Colon Adenocarcinoma Cell Lines

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Abstract. *Colchicum troodi* belongs to Colchicaceae family that particularly rich in flavonoids, phenolic acids, tannin, fatty acids and alkaloids such as colchicine. The aim of this study was to investigate the anti-proliferative and protective effects of *Colchicum troodi* ethanolic extract toward relevant molecular signalling pathways on Colo-320 primer and Colo-741 metastatic colon adenocarcinoma cell lines. *Colchicum troodi* was collected and extracted. Different concentrations of *Colchicum troodi* extract were incubated for 24 h and 48 h with Colo-320 and Colo-741 cells. Cell growth and cytotoxicity were measured by MTT assays. Anticancer and antiprolifetarive activities of *Colchicum troodi* were investigated by immunocytochemistry using antibodies directed against to β-catenin, LGR5, jagged 1, IHH, CD133 and Ki-67 in Colo-320 and Colo-741 cells. In the MTT assay, 10 μg/ml and 5 μg/ml *Colchicum troodi* extract were found to be active against Colo-741 and Colo-320 cells, respectively. *Colchicum troodi* extract significantly increased caspase β-catenin immunoreactivities while IHH immunostaining intensity was decreased in Colo-741 cells. We conclude that *Colchicum troodi* extract increased β-catenin via Wnt/β-catenin pathway in Colo-741 cells. Interestingly, it decreased IHH immunoreactivities which is an antagonist of constitutive activity of Wnt/ β-catenin signaling and is assumed to play protective act during carcinogenesis.

Key words: *Colchicum troodi*, colon, cancer, signaling pathways

Introduction

Colorectal cancer (CRC) is one of the most common cancer types and is the progress of cancer from the epithelial cells lining of the gastrointestinal tract (1). The epithelial outer surface of the intestine replace with the new cells in every 2-7 days and regeneration is maintained by self-renewing stem. This turnover of the epithelial cells occurs along the vertical axis (crypt-to-luminal surface) of the intestine. Progenitor stem cells differentiate to epithelial cells then migrate from the crypt base to the luminal surface. Any genetic mutations and epigenetic changes may be cause misregulation of the proliferation and differentiation steps and also could lead to uncontrolled cell dividing and tumor formation in gastrointestinal tract (2). Cancer cells populations in colorectal cancer are heterogeneous at the genetic, epigenetic, and phenotypic levels and including immune cells, pericytes, fibroblasts, endothelial cells and mesenchymal stroma/cancer stem cells (CSC). Especially, cancer stem cells are minor population of cancer, however are closely related to drug resistance and recurrence after primary treatment and tumor metastasis (3).

Multiple signaling pathways such as Wnt/β-catenin, Notch, TGF-β and Hedgehog signaling that regulate cell proliferation, stemness, differentiation and apoptosis in the intestinal vertical axis progression (4, 5). Also, the abnormalities of these signaling pathways are addressed in maintenance and self-renewal of cancer stem cells. In colorectal cancer, the mutations in these pathway signaling molecules initiate tumorigenesis and transform to malignant form. For this reason, the control of the signaling pathways is therapeutic target and has potential value in the treatment of colorectal cancer. In the intestine, Wnt/β-catenin signaling pathway is mainly active in the bottom of the crypt cells which have stem cell properties and amplify cell proliferation (6). In this pathway, β-catenin accumulation and degradation in the cytoplasm is controlled by a multimeric protein complex including of adenomatous polyposis coli (APC), axis inhibitor (Axin), casein kinase 1 (CK1), protein phosphatase 2 A and glycogen synthase kinase-3 beta (GSK3β). Hyperactivation of Wnt signaling pathway elevates β-catenin transcription factor level which increased expression of target genes such as c-myc and cyclin D and LGR5. Especially, LGR5 is a well-known intestinal stem cell marker in colorectal cancer (7). Another important signaling pathway is Notch which is involved in the cell division and control of stem cells. Notch signaling pathway is activated when jagged receptors on one cell interact with Notch transmembrane receptors on an adjacent cell (8). Additionally, indian hedgehog (IHH) is signal in a paracrine manner which is secreted by the differentiated epithelial cells to act on mesenchymal cells and is negative regulator of Wnt signaling. Differentiated enterocytes are expressed indian hedgehog in the small intestine and colon epithelium and controls tissue polarity (9).

Colchicum is a perennial flowering plants which belongs to Colchicaceae family. It grows from bulb-like corms and contains around 160 species. *Colchicum* is distributed Western Cape, parts of the Mediterranean coast, West Asia, Europe and down the East African coast to South Africa. Three genus of *Colchicum* grow in Cyprus; *Colchicum troodi* Kotschy, *Colchicum stevenii* Kunth and *Colchicum pusillum* Sieber, respectively. Specially, *C. toodi* is a genus of *Colchicum* and grows wide spread in Southern Cyprus. *Colchicum* species have flowers and leaves and are autumn-flowering species which are taxonomically very difficult group (10, 11).

Colchicum species are medical value plants which are used as medication for many diseases (12). Some of *Colchicum* species are cultivated for drug produce in pharmaceutical industry. *Colchicum* species contain flavonoids, phenolic acids, tannin, fatty acids and are rich in alkaloids such as colchicine (13). Colchicine is an alkaloid and has a therapeutic window through its anti-inflammatory, anti-mitotic and anti-fibrotic properties. The pharmacotherapeutic mechanism of colchicine is not fully understood in diverse disorders. Colchicine has been used for gout treatment for more than a millennium as well as is used for Behcet's syndrome, psoriasis, cirrhosis, amyloidosis and Familial Mediterranean Fever. Recent investigations have been demonstrated novel utilization of colchicine in oncology, immunology, cardiology and dermatology (14). Anticancer, anti-proliferative, anti-neoplastic, anti-inflammatory and muscle activity of colchicine derivatives were demonstrated by two US patents (15, 16). Additionally, colchicine has been used especially for the treatment of different cancer types such as myeloid leukemia and Hodgkin's syndrome (17).

The chemical compositions and cytotoxic activities of diverse *Colchicum* species were reported previously (18-21). According to literature only one study address the anti-cancer effects of *Colchicum pusillum* via Wnt/β-catenin pathway was demonstrated in colon cancer cell lines. Recent study suggested that *Colchicum pusillum* extract had toxic effects on Colo-320 cells and increased β-catenin and LGR-5 via Wnt/β-catenin pathway in Colo-741 cells (22). To our knowledge, there are no *in vitro* or *in vivo* studies addressing the anti-cancer effect of *Colchicum troodi* extract on any type of cancer. The specific effects of *Colchicum troodi* extract with respect to signaling pathway molecules in colon carcinoma cells remain undefined. Thus, we aimed to determine the Colchicum troodi extract anti-proliferative and protective effects toward relevant molecular signalling pathways, including β-catenin, LGR5, jagged 1, IHH, CD133 and Ki-67 which plays a role during carcinogenesis and transcription in primary (Colo-320) and metastatic (Colo-741) colon adenocarcinoma cell lines.

Materials and Methods

Plant material and extraction

The bulbs of *Colchicum troodi* Kotschy were collected in October 2017 from Trodos Mountains, Southern Cyprus. The plant is identified by Prof. Dr. Ali Hikmet Meriçli. Voucher specimen was deposited in the Herbarium of Near East University with the number NEUN 6904. 10 g quantity of the powdered *Colchicum troodi* bulbs was extracted with EtOH using Soxhlet apparatus for 3h, than EtOH was evaporated using with rotary evaporator till dryness. The extract of *Colchicum troodi* was dissolved in tertiary butanol-water in the ratio of 1:3.

Cell line and cell culture

Human colon adenocarcinoma cell lines Colo-320 (ATCC: CCL-220.1) and Colo-741 (ECACC: 93052621) were used in this study. Colo-320 and Colo-741 cells were cultured in media containing RPMI-1640 medium (Biochrom, FG 1215) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Capricorn Scientific, FBS-11B), 1% penicillin-streptomisin (Biochrom, A2213) and 1% glutamine (EMD Millipore, K0282). Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 and passaged when cultured cells reached confluency state. They were sub-cultured using 0.25% trypsin-EDTA solution (Biochrom, L 2143).

Cell viability assay

The cell viability was performed using a colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Glentham Life Sciences, GC4568). Stock *Colchicum troodi* extract (200 mg/ml) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Then, stock solution was diluted in culture medium with five different concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml). Cells (5 x 10^3/ml) were seeded into each well of 96-well culture plates in 100 µl medium. Negative control row neither contained any cells nor *Colchicum troodi* extract and positive control row only had seeded cells. *Colchicum troodi* extract dilutions were triplicated. Colo-320 and Colo-741 cell lines were incubated for 24 and 48 h. After incubation, 10 µl MTT solutions were added to the each well and reactions were done at 37°C for 4h. After incubation, 50 µl DMSO was added to dissolve the formazan salts. The absorbance was measured at 540 nm with spectrophotometer (Versa Max, Molecular Device, Sunnyvale, USA).

Immunocytochemistry

To evaluate cell responses to *Colchicum troodi* extract, cultured Colo-320 and Colo-741 cells were assessed immunocytochemically for binding of antibodies against β-catenin, leucine-rich repeat containing G protein-coupled receptor 5 (LGR5), jagged 1, indian hedgehog (IHH), CD133 and Ki-67. Colo-320 and Colo741 cells were fixed with 4% paraformaldehyde in PBS at 4°C for 30 minutes and then tween 20 (Sigma-Aldich) was added for permeabilization for 15 minutes. The cells were washed with PBS. Incubation with 3% H_2O_2 for 5 minutes, endogenous peroxidase activity was quenched. Then, cells were washed (x3 times) with PBS. For overnight incubation at 4°C, primary antibodies β-catenin (sc-59737, Santa Cruz Biotechnology), LGR5 (HPA012530, Sigma Prestige Antibodies), jagged 1 (sc-8303, Santa Cruz Biotechnology Inc.), IHH (sc-13088, Santa Cruz Biotechnology Inc.), CD133 (MAB4310, Merck Millipore) and Ki-67 (RB-081-A1, Thermofisher Scientific) were added. Biotinylated secondary antibody (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) was added and incubated for 30 minutes. Then, cells were washed (x3 times) with PBS. Streptavidin-peroxidase complex (100 µl) was added to cultured cells. Cells washed (x3 times) by PBS. Then, DAB was added and incubated for 5 minutes for enhancement of immunolabeling and cells were washed with distilled water. Cells were counterstained with Mayer's hematoxylin for 5 minutes and mounted with mounting medium (Merck Millipore, 107961, Germany). All specimens were examined using a light microscope (Olympus BX40, Tokyo, Japan).

Staining of β-catenin, LGR5, jagged 1, IHH, CD133 and Ki-67 was also graded semi quantitatively using the H-SCORE. It was calculated with the

following equation: HSCORE=Σл (i+1), where i is the intensity of staining with a value of 1, 2 or 3 (mild, moderate, or strong, respectively). л is the percentage of cells stained with each intensity, varying between 0 and 100%.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). The results were analyzed using GraphPad Prism 7 software. Differences among groups were analyzed statistically with Mann-Whitney where appropriate. A p value of <0.05 was considered as statistically significant.

Results

Cell viability and cytotoxicity

Cell viability was determined by MTT assay and seeding Colo-320 and Colo-741 cells were incubated with different concentrations of (5, 10, 20, 50 and 100 µg/ml) *Colchicum troodi* extract for 24 and 48 hours. Our results reported that, *Colchicum troodi* extract at 10 µg/ml concentration was more effective at inhibiting Colo-741 cell growth for 48 h when compared with other concentrations and incubation periods. For Colo-320 cell line, 5 µg/ml *Colchicum troodi* extract was used at for evaluate anticancer activities for 24 h. Based on results from MTT assay, all concentrations of *Colchicum troodi* extract decreased dramatically Colo-320 cells proliferation and had toxic effects for 48 h incubation period (Figure 1).

Cell morphology

Colo-741 cells have typical fibroblast colony morphology in standard culture conditions. After treated with *Colchicum troodi* extract for 48 hour, the shapes of Colo-741 cells changed to oval and vacuoles were detected in the cytoplasm (Figure 2 A, B). In standard culture conditions, Colo-320 cells are semi-adhesive, refractile and rounded cells. After incubation with *Colchicum troodi* extract for 24 h, the number of the Colo-320 cells was decreased and vacuoles were detected in the cytoplasm.

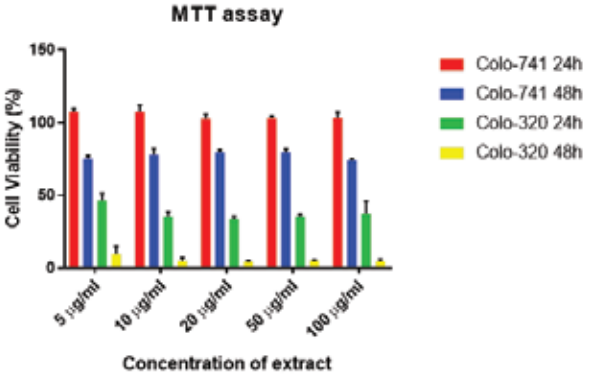


Figure 1. MTT assay results. Effect of *Colchicum troodi* extract on cell viability of Colo-320 and Colo-741 cells. Both Colo-320 and Colo-741 cells were treated at different concentrations (5, 10, 20, 50 and 100 µg/ml) for 24 and 48 h.

Immunohistochemical assessment

Strong β-catenin immunostaining was detected in *Colchicum troodi* extract treated both Colo-741 and Colo-320 cells (Figure 3 A) (Figure 4 A). The H-SCORE for β-catenin was significantly higher in treated Colo-741 cells than control group (p>0.05, Table 1). Additionally, the intensity of β-catenin was a significantly different between *Colchicum troodi* extract treated Colo-741 (Figure 3 A) and Colo-320 (Figure 4 A) cells (p<0.05, Table 1).

LGR5 immunoreactivity was detected in *Colchicum troodi* extract treated Colo741 and Colo-320 cells and control groups (Figure 3 C, D) (Figure 4 C, D). However, *Colchicum troodi* extract treated Colo-741 cells was shown significant decrease in H-SCORE when compared with *Colchicum troodi* extract treated Colo-320 cells (p<0.05, Table 1).

Immunostaining intensity for IHH was strong in *Colchicum troodi* extract treated Colo-741 cells (Table 1). After H-SCORE analyses, IHH immunoreactivity was significantly higher in *Colchicum troodi* extract treated Colo-741 cells (Figure 3 G) than Colo-741 control group (Figure 3 H) (p<0.05, Table 1). Immunoreactivity for IHH was similar in both *Colchicum troodi* extract treated Colo-320 cells and its control group (Figure 4 G, H).

The immunostaining intensity of CD133 was moderate in Colo-741 cells after incubation with

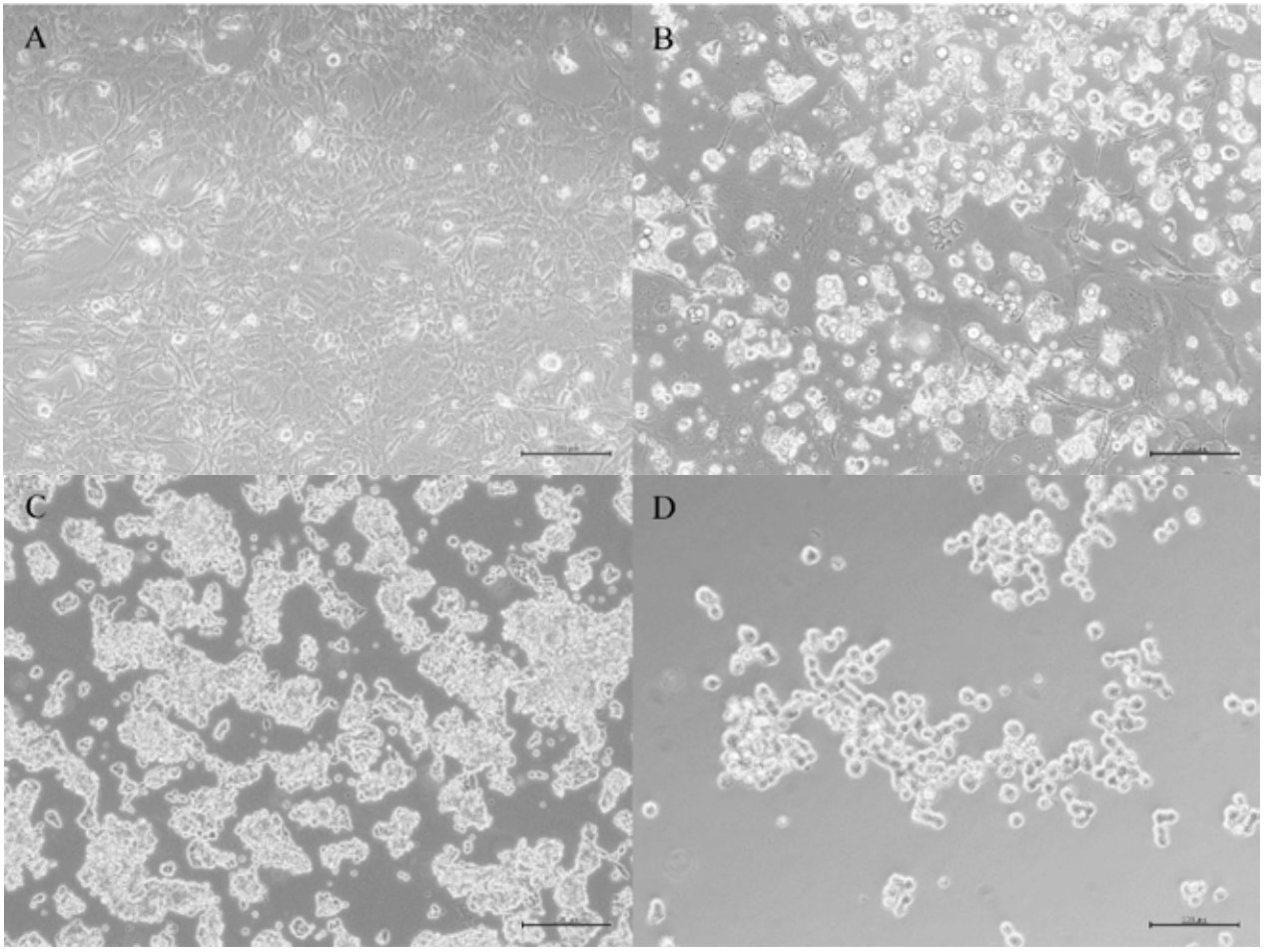
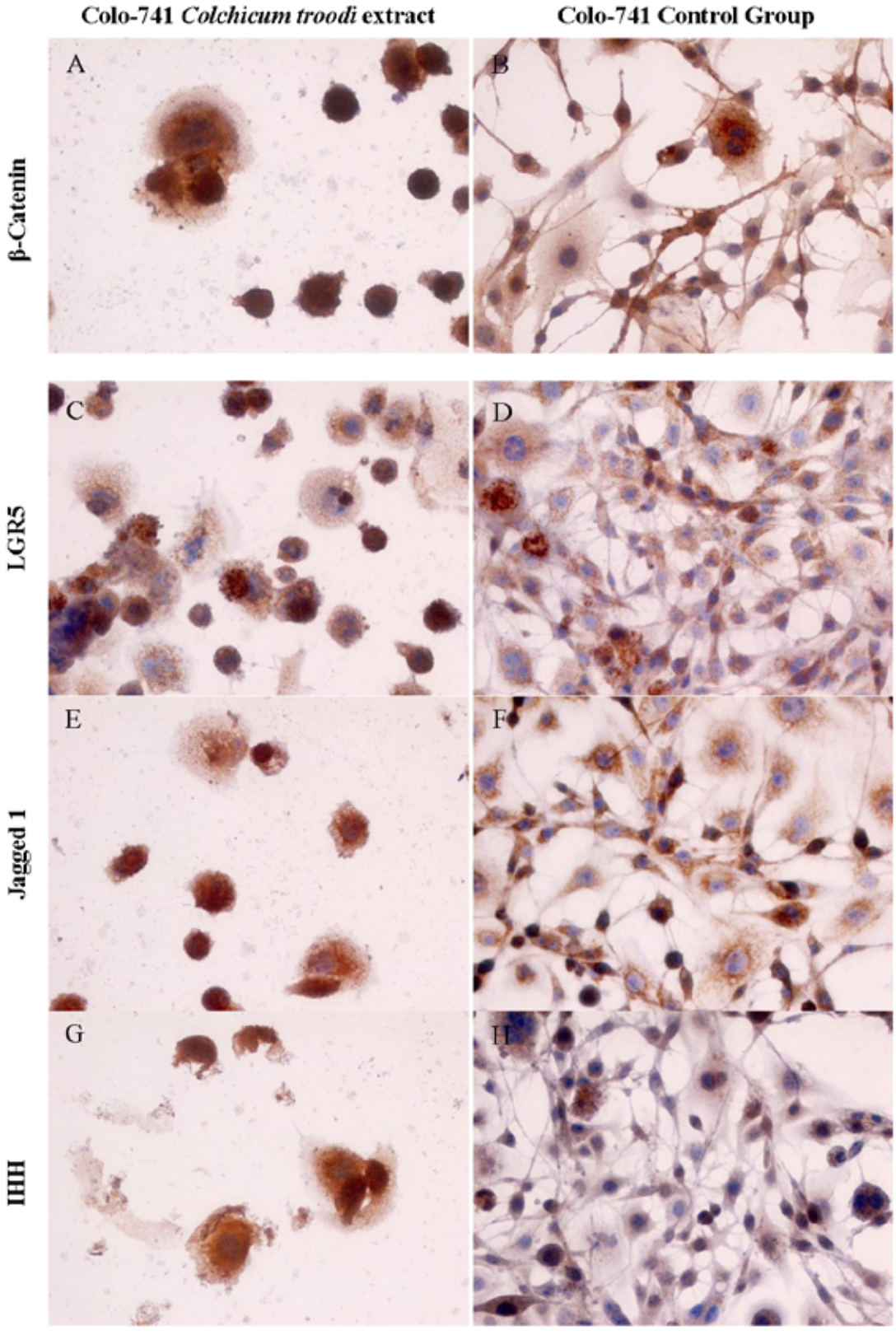


Figure 2. Colo-741 (A, B) and Colo-320 (C, D) cells imaged under the inverted microscope. Control group Colo-741 cells (A), *Colchicum troodi* extract treated Colo-741 cells (B), control group Colo-320 cells (C) and *Colchicum troodi* extract treated Colo-320 cells (D). Scale bars= 200 μm.

Table 1. The H-SCORE of β-catenin, LGR5, Jagged 1, IHH, CD133 and Ki-67 in Colo-320 and Colo-741 cells treated with Colchicum troodi extract for 24 and 48 h, respectively.

	Colo-741 *Colchicum troodi*	**Colo-741 Control**	**Colo-320 *Colchicum troodi***	**Colo-320 Control**
β-catenin	382±12.81[a,c]	281.4±47.83	364.4±9.83	320.8±46.15
LGR5	317±18.89[d]	296.4±45.82	383.2±23.69	364.4±22.3
Jagged 1	339.8±37.67	306.4±25.19	368.4±24.8	325±25.32
IHH	354.9±18.43[b]	189.6±27.98	360.4±9.127	347.2±28.96
CD133	317.5±71.59[e]	316.9±35.02	379.8±14.36	374.4±13.11
Ki-67	362±11.55	341.4±29.62	370±27.29	349.6±19.63

Data is expressed as means ± SD and were compared by Mann-Whitney.
[a,b] The data was significant when compared with Colo-741 control group ($p<0.05$).
[c,d,e] The data was significant when compared with Colo-320 cell lines ($p<0.05$).


Colo-741 *Colchicum troodi* extract
Colo-741 Control Group
β-Catenin
LGR5
Jagged 1
IHH
A
B
C
D
E
F
G
H

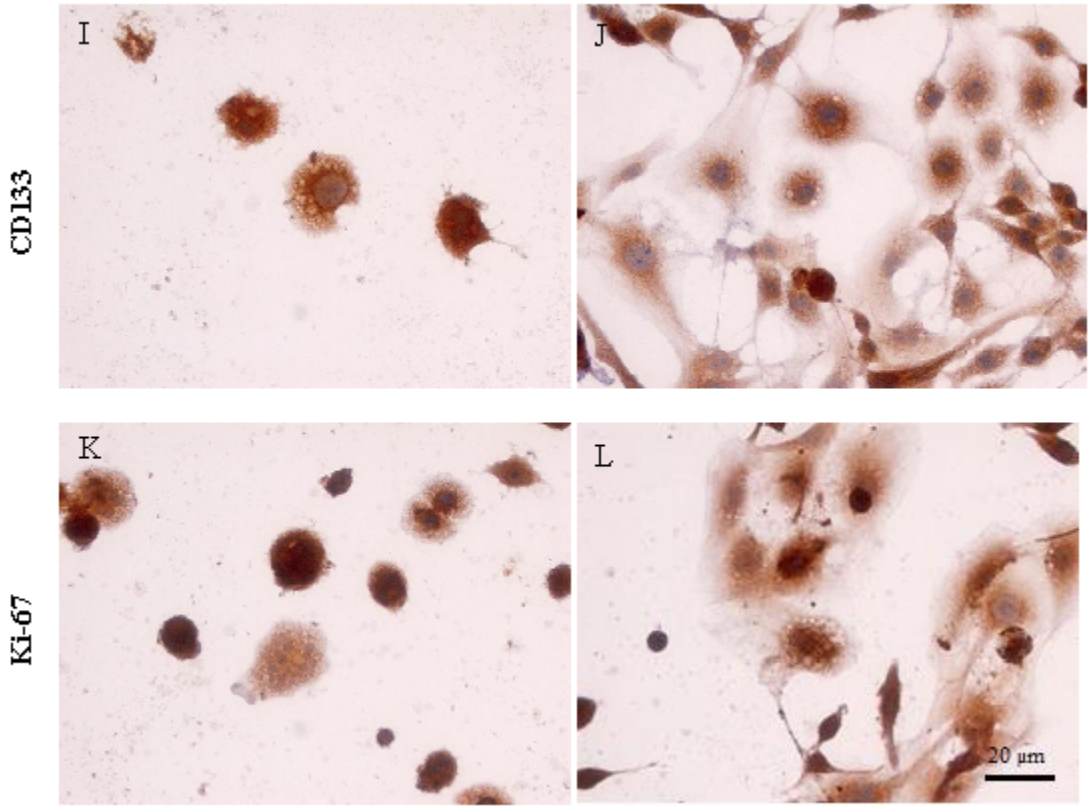


Figure 3. Immunoreactivity of β-catenin, LGR5, Jagged 1, IHH, CD133 and Ki-67 in Colo-741 cells in 48 h culture exposed to standard culture conditions (B, D, F, H, J, L) or 10 µg/ml *Colchicum troodi* extract (A, C, E, G, I, K). (Scale bars= 20 µm)

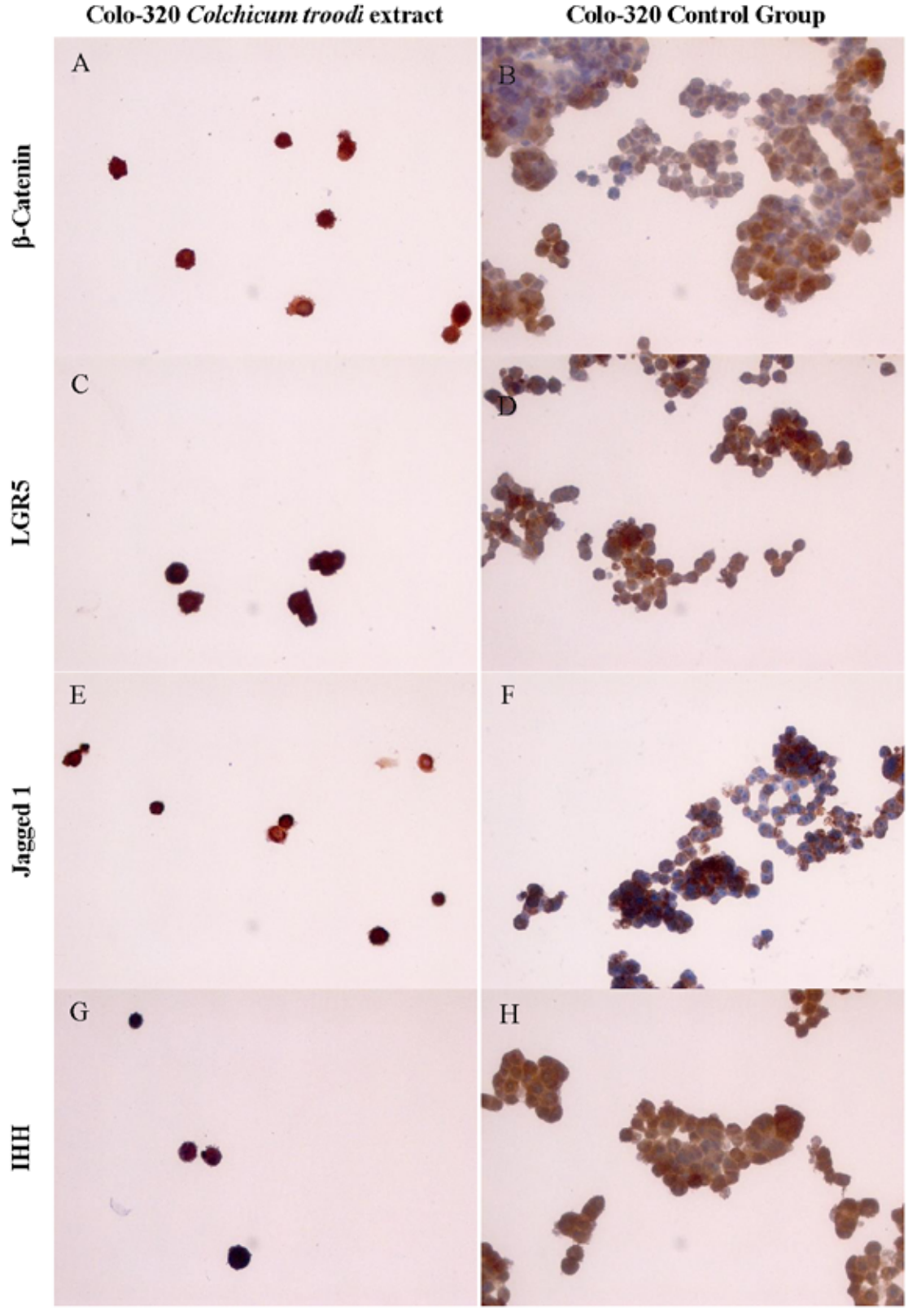
Colchicum troodi extract for 48 h (Figure 3 I). The CD133 immunoreactivity was significantly lower in *Colchicum troodi* treated Colo-741 cells (Figure 3 I) than in *Colchicum troodi* treated Colo-320 cells (Figure 4 I) ($p<0.05$, Table 1).

As shown in Figure 3, jagged 1 immunoreactivity was similar in all groups. The increased H-SCORE values were calculated for *Colchicum troodi* extract treated Colo-741 and Colo-320 cells than control groups, but this increase was not significant for both types of the cell lines ($p>0.05$, Table 1).

Additionally, similar and not significant Ki-67 immunoreactivities were detected in all groups (Figure 3K,L and 4K,L), ($p>0.05$, Table 1).

Discussion

Tropolone alkaloids content of *Colchicum* species has been widely used in pharmaceutical industry for many years and still remains important in treatment of gout, Behcet's disease, Familial Mediterranean Fever, amyloidosis, Hodgkin lymphoma, skin cancers and myeloid leukemia. Recent years, studies reported that the different *Colchicum* species have cytotoxic activities because of their chemical constituents (18-21). Moreover, the effects of *Colchicum pusillum* on Wnt/β-catenin signaling pathway in colon adenocarcinoma cells were shown by our study group before (22). To date, there is no study investigating the effects of *Colchicum troodi* with respect


Colo-320 *Colchicum troodi* extract
Colo-320 Control Group
β-Catenin
LGR5
Jagged 1
IHH
A
B
C
D
E
F
G
H

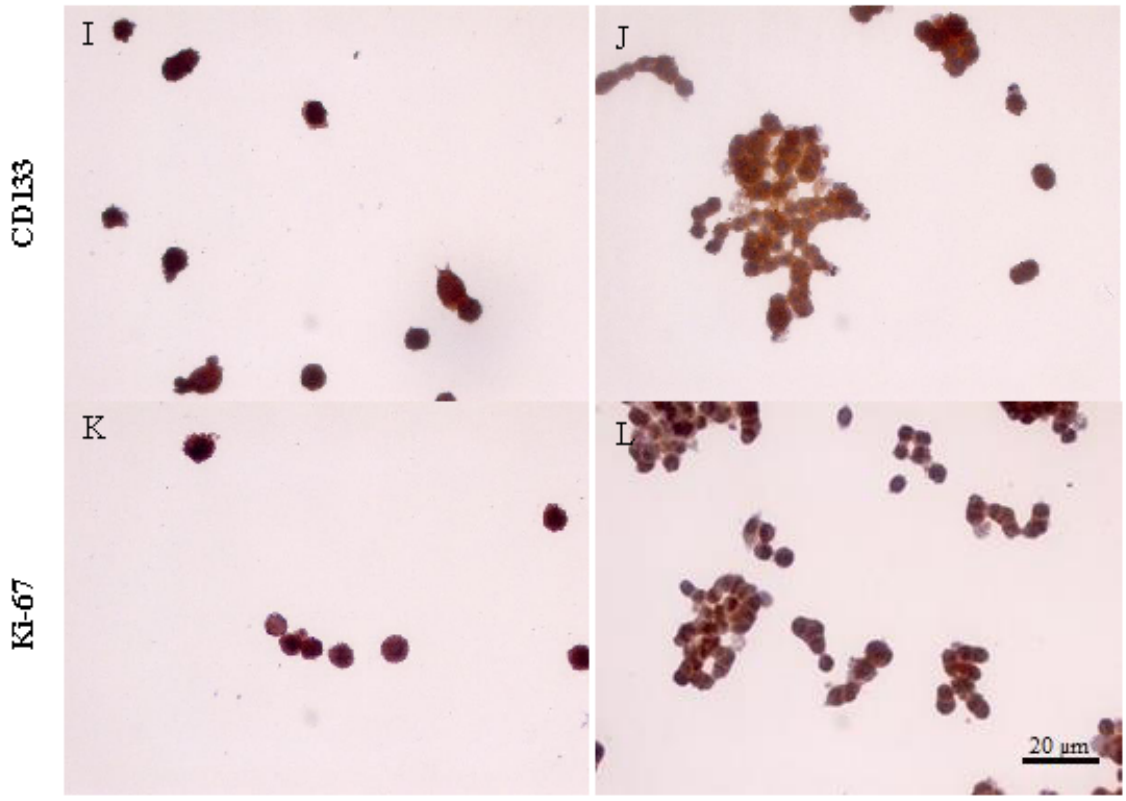


Figure 4. Immunoreactivity of β-catenin, LGR5, Jagged 1, IHH, CD133 and Ki-67 in Colo-320 cells in 24 h culture exposed to standard culture conditions (B, D, F, H, J, L) or 5 µg/ml *Colchicum troodi* extract (A, C, E, G, I, K). (Scale bars= 20 µm)

to Wnt/β-catenin, Notch and Hedgehog pathway molecules in colon adenocarcinoma cells. Our results showed that β-catenin, IHH and LGR5 immunoreactivities were significantly increased after treated with *Colchicum troodi* extract in metastatic colon adenocarcinoma (Colo-741) cells at 10 µg/ml concentration for 48 h.

Colchicine is an alkaloid which obtained from *Colchicum* species and blocks mitosis by inhibition of microtubule formation (14). Cells sensitivity to colchicine is different at the stages of mitosis. At higher concentrations of colchicine, metaphase of mitosis was blocked immediately. On the other hand, at colchicine lower concentration, cells were more sensitive and were blocked in prophase phase (23). In our previous study, we showed that *Colchicum pusillum* extract increased Ki-67 levels in Colo-741 cells. In the current study, *Colchicum troodi* extract altered cell morphology and decreased cell number in both Colo-320 and Colo-741 cells. However, Ki-67, cell proliferation marker, was increased in *Colchicum troodi* extract treated Colo-320 and Colo-741 cells but results did not significantly different. Ki-67 is a nuclear antigen and is expressed in G1 to M-phase, interphase, of the cell cycle (24). The blocking of the prophase or metaphase phase of the cell division by colchicine may be the reason of the unchanged and increased Ki-67 levels in *Colchicum troodi* treated Colo-320 and Colo-741 cells. The cells may respond as proliferate after *Colchicum troodi* tretatment. However, the proliferation and inhibition of the both Colo-741 and Colo-320 cells was balanced during administration of the *Colchicum troodi*, therefore, differences were not observed. We speculated that the cell morphology changing may be related to disrupting of microtubules which are found in the cell cytoskeleton by colchicine.

The Wnt/β-catenin signaling pathway is one of the most crucial and complex pathways in tumorigenesis and progression of colorectal cancer. It regulates β-catenin level which is used in coordination of cell-cell adhesion, chromatin interactions and gene transcription of target gene for epithelial proliferation (6). In recent study, it was shown that *Colchicum pusillum* extract has stimulant effects on Wnt/β-catenin pathway by increasing β-catenin immunoreactivity in Colo-741 cells (22). In our study, we reported that the immunoreactivities of β-catenin in *Colchicum troodi* extract treated Colo-741 cells were significantly higher than control group. Also, the immunoreactivities of β-catenin in *Colchicum troodi* extract treated Colo-741 cells were significantly higher than *Colchicum troodi* extract treated Colo-320 cells. It can therefore be concluded that metastatic cancer cells may have more resistant to *Colchicum troodi* extract administration throughout Wnt signaling pathway. Also, primary and metastatic colon cancer cells were expected different response to different treatment such as *Colchicum troodi*.

Epithelial turnover of intestine depends on a self-renewing stem cell population in crypt base. Rapid proliferation and expansion of stem cell is followed by migration of differentiated daughter cells from crypt base to the luminal surface. In colorectal cancer cells, hyperactivation of Wnt pathway increase stem cell-associated genes expressions such as LGR5 gene. LGR5 is found in crypt base columnar cells and is a stem cell marker which is a primary marker of colorectal cancer (25). Furthermore, numerous cell surface markers are associated with stemness characteristics of colorectal cancer stem cells such as CD133. High CD133 and LGR5 expression are associated with poor prognosis and metastasis in colorectal cancer (26). Considering our results, which showed that LGR-5 and CD133 immunoreactivities were not significantly different in *Colchicum troodi* extract treated Colo-320 and Colo-741 cells than both control groups. However, LGR-5 and CD133 immunoreactivities were significantly higher in *Colchicum troodi* extract treated Colo-320 cells than *Colchicum troodi* extract treated Colo-741 cells. The results indicated that *Colchicum troodi* extract may be more effective in promoting stemness properties of primary colon adenocarcinoma cells. In addition, metastatic cancer cell pool could contain different types of cancer cells, therefore treatment effect may be insufficient for them.

Indian hedgehog pathway is an antagonist of constitutive activity of Wnt/ β-catenin signaling during colorectal tumorigenesis (27). In many studies reported that downregulation of *indian hedgehog* signaling pathway has been observed in an early phase of colorectal cancer formation (28, 29). Additionally, Gerling et al. (2016) showed that indian hedgehog is mainly expressed in stromal tissue and can be used as a tumor suppressor and, also elevated expression will inhibit colorectal cancer (30). The inhibitory function of *indian hedgehog* signaling pathway in tumorigenesis of colorectal cancer is still not clear. However, various studies also confirmed that the activation of indian hedgehog might contribute to the downregulation or loss of Wnt expression and may work against colorectal cancer (31, 32). Interestingly, our results showed that the immunoreactivity of indian hedgehog (IHH) was significantly increased in Colo-741 cells incubated with *Colchicum troodi* extract compared to control group. These data raised a hypothesis that *Colchicum troodi* extract may be have protective effects by upregulating IHH expression in colorectal cancer.

In conclusion, we demonstrated that cytotoxic and anti-cancer effects of *Colchicum troodi* extract in both primary (Colo-320) and metastatic (Colo-41) colon carcinoma cells using different concentrations. Interestingly, *Colchicum troodi* extract increased β-catenin expression which is a transcription factor and promotes cell proliferation in Colo-741 cells. On the other hand, IHH, antagonist of Wnt/ β-catenin pathway, expression was elevated in *Colchicum troodi* treated Colo-741 cells. These results indicate that *Colchicum troodi* extract maybe has protective activity through IHH signaling pathway. *Colchicum troodi* extract contains different chemical compounds, it is not the colchicine alone. Therefore, in order to resolve exact anticancer effects of *Colchicum troodi* on colon cancer signaling pathways, active chemical composition must be identified. Further assessment of active chemical compounds of *Colchicum troodi* extract with different signaling pathway molecules that includes all possible tumorigenesis and metastasis mechanisms, is necessary.

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Declaration of competing interest: The authors declare that they have no conflict of interest.

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