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ORIGINAL ARTICLE

Effects of *Colchicum baytopiorum* leaf extract on cytotoxicity and cell death pathways in C-4 I and Vero cell lines

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Summary

Purpose: *The present study aimed to research the cytotoxic effects of Colchicum baytopiorum extract on normal and cancerous cells and reveal the cell death mechanisms in cancerous cells triggered by this effect.*

Methods: *Within this framework, the cells' index values obtained with an xCELLigence Real Time Cell Analysis DP device, selectivity index (SI), apoptotic index (AI) based on a DAPI application and time-related activities of caspase 3,7 and 9 with a spectrofluorometer were inspected. The expressions of apoptosis/autophagy/entosis/necroptosis/anoikis-related genes were researched with qRT-PCR.*

Results: *It was determined that C. baytopiorum extract had displayed a high selectivity [(SI)=4], increased AI ($p<0.01$) and activation of caspases 3,7 and 9 ($p<0.05$). It was observed that the mRNA expressions of Atg12, Atg16, Atg5, Atg7, bad, bak, bax, bcl-xL, Beclin1, caspase3, FLIP, Puma, LC3, mcl-1, Bit1, Rho, RIP1, ROCK and TRAF2 genes in C-4 I cells to which the plant extract was applied had increased significantly in comparison with the control group ($FC\geq1.5$). A lowering was detected in the mRNA levels of IAP, SRC kinase and TNF.*

Conclusion: *Consequently, it was revealed that the plant extract used had increased the gene expressions in the autophagic cell death pathway in C-4 I cells along with apoptosis and thus, it could be a promising candidate for cervix carcinoma treatment.*

Key words: *C. baytopiorum, cell death, cytotoxicity, C-4 I cells, Vero cells, xCELLigence*

Introduction

Cancer can be defined as a genomic disease resulting from dynamic changes in the DNA of an organism. Today, the use of natural products in the treatment of cancer is of high importance. The potential anti-tumor effects of many plants have been revealed in recent studies [1]. Additionally, the anti-tumor effects of some plants has not been scientifically proven. For this reason, any studies conducted in this respect with such plant extracts that are endemic and whose biological activity has not been revealed yet are highly important for consciously using natural medicines and for the potential of developing new medicines.

The existence of colchicine, 2-dimethylcolchicine, 3- dimethylcolchicine, 3-dimethyl-N-formyl-N-deacetylcolchicine, 2-dimethyl- γ-lumicolchicine, 2-dimethylcolchifolin, demecolcine, 2-dimethyl demecolcine, cornigerine, N-formyl-N-deacetylcolchihine and 3-dimethyldemecolcine alkaloids is known in the *C. baytopiorum* plant, endemic in Turkey, which was used in our study. Furthermore, it consists in total of 11 phenolic acids which are namely benzoic, coumaric, vanillic, cinnamic ferrulic, caffeic acids, 2-hydroxy-6-metoxybenzoic acid, 4-hydroxy-3-metoxybenzoic acid, 2,5-dihydroxybenzoic acid, 2-hydroxybenzoic acid and 4-hydroxybenzoic [2].

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It was shown in various studies that raw extracts of natural products contained a variety of molecules with anti-tumor effects and some of them were very effective in killing cancer cells [3,4]. However, there are some other studies claiming that the use of total plant extracts instead of a single substance obtained from plant extracts is more effective in some cases, due to the complex interactions (synergetic and/or antagonist effects) of all compounds in the plant extracts [5-7].

It is considered useful for researching the chemotherapeutic targets of cancer that the response of anticancer medicines to cancer cells is observed and it is ensured that they are directed to the cell death pathways such as apoptosis, autophagy, anoikis, entosis, necroptosis etc. with the increase of the effect and the joint molecular path where the pathways followed by these types of death are determined [8,9]. The fact that the various agents developed increases the sensitivity of tumour cells to apoptosis can be given as an example of this situation [10].

The pathways associated with autophagic cell death are also targeted for the development of a cancer treatment. Autophagy causes cell death when it is activated excessively in the cell. Today, it is accepted that the signal pathways of PI3KCI-AKT-mTORC1 are key regulators for the cellular processes where restrictions can be eliminated with various genetic and epigenetic mechanisms in cancer cells. PI3K-Akt pathways regulate mTORC1 activity through oncoproteins and/or loss of tumour repression. In this way, mTORC1 is frequently seen in inhibited autophagy malignant cells [11].

When the caspase activation is blocked, it can represent an alternative cell death mechanism so that necroptosis will replace apoptosis. It is reported that different physical-chemical stress stimulants such as ionizing radiation, photodynamic therapy, excessive calcium and glutamate including anticancer medicines can initiate necroptosis [12].

The cell structures that are formed through entosis in various human cancers like breast, colon, pancreatic carcinoma etc. have been identified [13]. Similarly with the entotic cell structures, it is known that ingested cell structures in human tumours involve complicated and multiple cell relationships such as cell in cell, another cell therein etc. So, the ingestion of live tumour cells in human tumours is generally defined as cell cannibalism and shaped with various mechanisms like entosis. Cell in cell structures have recently been defined in primary breast tumours in addition to metastatic cells [14-16].

Entosis assumes a potential role in tumour repression and restricts the growth of tumour cells which are subjected to a culture process in soft agar [16].

It is reported as a critical aspect in tumour cells that they gain an independent growth potential without binding. Normal epithelial breast cells that lack a matrix binding result in apoptosis or anoikis. However, tumour cells or oncogenic transformed cells refrain from this process and continue to reproduce [17]. Cancer cells continuously develop various mechanisms to prevent anoikis and help metastases in this way. Cancer cells adapt to the metastatic region and gain resistance to anoikis by achieving a special change in their integrins, reaching to epithelial mesenchymal connections; being used in the activation of primer vital signals due to internal and external signals; re-regulating or adapting their metabolisms through autophagy [18,19].

Information on the cytotoxic effect of the extract prepared with the leaf of the *C. baytopiorum* plant on normal (Vero) and cancer (C-4 I) cells is very limited. In this context, it was aimed to research the potential of the plant for use in cancer treatment. Furthermore, it was intended to obtain data about which cellular death mechanisms are triggered and how and whether these pathways are associated with each other or not. Consequently, it was targeted to identify the role of apoptosis/autophagy/entosis/necroptosis/anoikis encouraged by the cytotoxic effect mechanism at the cellular and molecular level and make substantial contributions to the potential treatment methods for cancer and planning of new treatment methods related thereto and the development of new plant medicines.

Methods

Preparation of the Colchicum baytopiorum plant extract

The parts of the *C. baytopiorum* C.D. Brickell (leaf, ISTE: 81438) plant collected from the Antalya Termessos district that were used in the experiments were separated and sliced. Afterwards, the plant materials were dried in an incubator at 60°C and powdered and then percolated with pure methanol. The obtained methanol extracts were lyophilized by means of concentrating them in a rotary evaporator at 30-35°C. The lyophilized extracts were dissolved in distilled water and 2x serum-free media (Waymouth for C-4 I cell and MEM for Vero cell) were added and subjected to sterile filtration and the stock solution was obtained. The prepared stock solution was diluted with a sterile medium containing 10% FBS and it was prepared at different concentrations (2.5, 1, 0.25, 0.1, 0.025, 0.01 mg/ml) [2].

Cell lines and cell culture

C-4 I and Vero cell lines were used in this study. The C-4 I (ATCC, CRL-1594) cell line is of cervix carcinoma origin. Waymouth Medium (W1625, Sigma) containing 10% fetal bovine serum (FBS, Gibco Lab), 100 IU/ml penicillin (Pronapen, Pfizer) and 100 µg/ml streptomycin (Streptomycin sulphate, İ.E. Ulagay) was used.

The Vero cell line (ATCC, CCL-81), used as a normal cell source, was obtained from green monkey (Cercopithecus aethiops) kidney in the 1960s. In order to grow Vero cells, MEM (Minimum Essential Medium, Sigma) containing 10% FBS (Gibco Lab), 100 IU/ml penicillin (Pronapen, Pfizer) and 100 µg/ml streptomycin (Streptomycin sulphate, İ.E. Ulagay) was used as a medium.

The cells were plated in 25 cm^2 culture plates by changing the medium 2-3 times a week in an incubator using 37°C, 5% CO_2 and 95% air [20,21].

Real time cell index measurement

Within the scope of the real-time cell index measurement with the xCELLigence RTCA-DP system, 100 µl of medium was added to each well of an E-Plate with 16 wells and the check value was obtained with the device. For C-4 I and Vero cells, each well was cultured, so that there would be 6000 cells in 100 µl of medium [22]. For the experimental group which would be used as a negative control only, no cell culture was performed and only the medium was added to the wells. E-Plates were kept in the sterile working cabinet for 20 min for the sake of cell adhesion and then the E-Plates were placed in the device. Measurements were taken on the device once every 15 min. Twenty h after the cell culture, the E-Plates were removed from the device and the medium on the cells was eliminated and the prepared plant extracts at different concentrations were applied. Only the medium was added for positive (the cells not subjected to the extract application) and negative control groups. After this process, the E-Plates were placed in the device again and left for incubation for 72 h and measurements continued to be taken once every 15 min. The impedance related cell index graphics were transferred to the monitor of the computer and the IC_{50} values of the plant extracts used were determined. Real-time cell death and proliferation were continuously measured with the use of the xCELLingence RTCA-DP system, and the obtained data were converted into a result graphic with the use of RTCA-DP software (Roche Diagnostics GmbH) [23].

Selectivity index (SI)

The selectivity index value was calculated according to the following formula for each plant extract:

$$SI = IC_{50} \text{ normal cell} / IC_{50} \text{ cancer cell}$$

When the high SI value of the used plant extracts was calculated to be >2, it was accepted that it had had a selective toxic effect on the cancer cells. An SI value of <2, on the other hand, showed that it displayed a general toxicity [24].

Apoptotic index (AI)

The cells were cultured in 12-well plates (150.000 cells/ml) and trypsinisated with 0.25% Trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc). The cells were fixed at room temperature with the use of methanol:FTS (1:1) and pure methanol respectively for 3 min. Following the fixation, the cells were subjected to DAPI (Sigma-Aldrich; Merck KGaA) application for 20 min and washing with PBS. The cells were evaluated under a fluorescent microscope [25]. The AI percent was determined with the use of the following formula [26]:

(apoptotic cell number/total cell number)×100

Caspase activation analysis

Caspase 3/7 and 9 test kit (CaspaTag™ Caspase-3,7 In Situ Assay Kit, Fluorescein Kat. No. 92590, Millipore) (CaspaTag™ Caspase-9 In Situ Assay Kit, Fluorescein Kat. No. APT409, Millipore) were used for determining the activations of caspase 3,7 and 9 in C-4 I cell cultures to which the *C. baytopiorum* plant extract was applied [25].

Real time polymerase chain reaction (qRT-PCR)

The cells in the experimental group and the control group (C-4 I), to which was added the IC_{50} concentration of the extract obtained from the *C. baytopiorum* plant, were subjected to total RNA insulation with the use of a total RNA insulation kit (Ambion PureLink RNA Mini Kit, Cat. No: 12183-018A) at the end of 24 h [27].

The obtained total RNA quantities were diluted so that they would be 100 ng/µl. Eleven samples were prepared for each experimental and control group. The cDNA kit protocol (Applied Biosystems, High-Capacity cDNA Reverse Transcription Kit, Cat. No: 4368814) was conducted to determine the extent of change in the gene expressions of total RNSs insulated from cell lines associated with apoptosis/autophagy/anoikis/entosis/necroptosis.

After the cDNAs were determined, qRT-PCR was applied. For each sample (the control group and experimental group to which the *C. baytopiorum* plant extract was applied), 3.9 µl nuclease free water, 5 µl Master Mix (Applied Biosystems, TaqMan Gene expression Master Mix, Cat No:4369016), 1.1 µl cDNA were added to the lyophilized plate (TaqMan Array 96-Well Fast Plate, Costom Format 45, Applied Biosystems) wells of the primer and probes. The samples were programmed as shown in Table 1 and then placed in the qRT-PCR device [27].

With the use of the qRT-PCR technique, the gene expressions, which played a role in the process associated with apoptotic/autophagic/entosis/anoikis/necroptosis, were observed. The expressions of bcl-xL, bid, bad, bax, bak, bcl-2, mcl-1, Puma, Noxa genes belonging to Bcl-2 gene family playing a role in apoptosis were also observed. Additionally, caspase 3, 8, 9 activation, Akt, TRAF2 and Fas that was in charge at the external pathway of apoptosis, FADD, TRADD, TNFα and TNFR1 expression level were also examined. Moreover, the expressions of bif-1 being a member of the Bcl-2

Table 1. Programme table of PCR apparatus which used for qRT-PCR

Affiliation and Pre-denaturation	*PCR amplification*
1 cycle:	40 cycles:
At 50°C for 2 min.	At 95°C for 15 sec.
At 95°C for 10 min.	At 60°C for 1 min.

gene family regulating autophagy and BNIP-3 proteins and Atg5, Atg12, Atg16, Beclin1 and LC3 proteins effective in autophagic cell death were searched, besides apoptotic cell death. The expressions levels of ERK1, Bim, Bit1, FAK, MAP kinase, ILK, Src kinase proteins associated with anoikis were assessed. With respect to entosis, the expressions of Rho, ROCK, Atg 5/7, LC3 proteins were examined. With respect to necroptosis, on the other hand, the expressions of RIP1, RIP3, CYLD, FLIP, IAP proteins were observed. GAPDH, HPRT1, 18S and GUSB were used as endogen. Each gene pair was restudied.

The results obtained from qRT-PCR were expressed with the comparative threshold (Ct) method [27]. The fold rate (fold change - FC - ≥ 1.5) value was calculated with the use of the expression rates of a gene [28].

Statistics

The values obtained from all experimental groups were subjected to a unidirectional ANOVA test. The significance of the groups in comparison with the control group was assessed with t-test. P<0.05 was assessed with GraphPad Prism version 6.00 (GraphPad Software, San Diego California USA).

Results

Determination of the IC_{50} concentrations and durations of the plant extracts

The Vero and C-4 I cell lines were cultured in 16-well plates so that there would be 6000 cells.

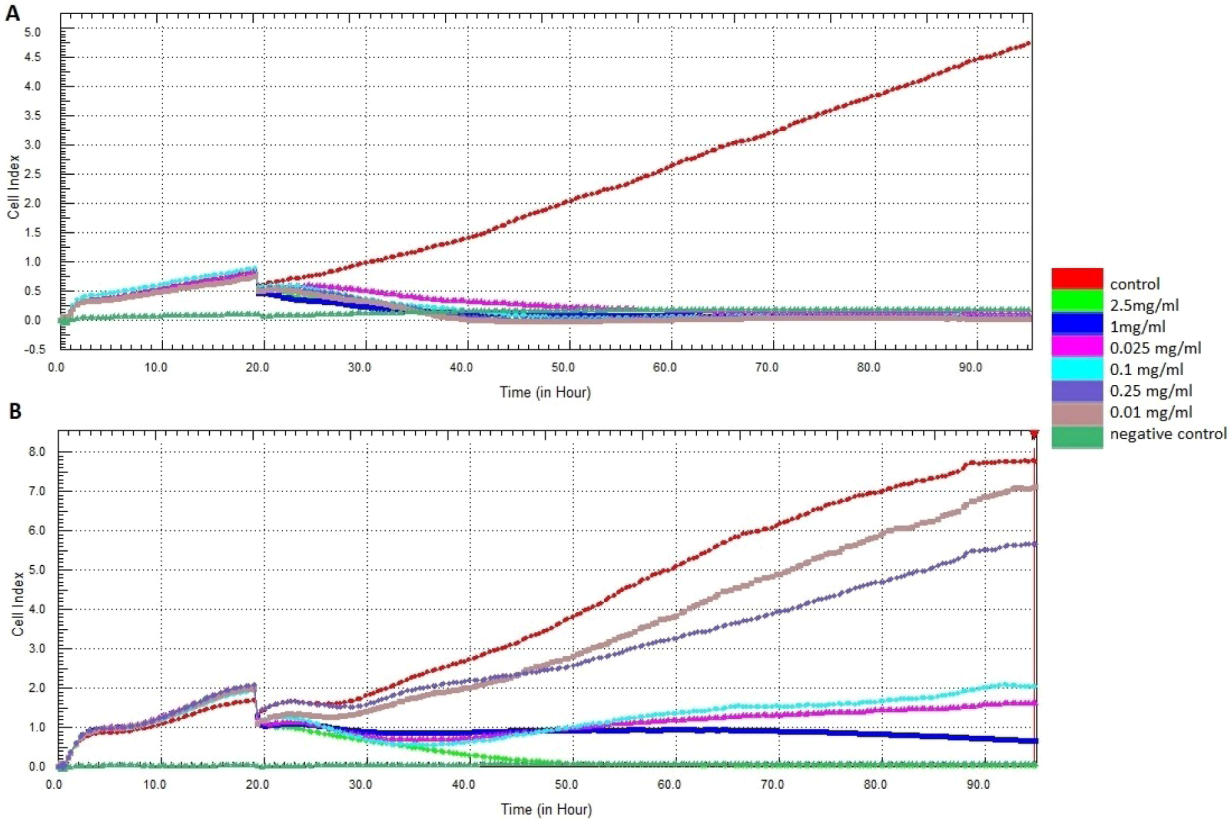

Figure 1. Cell index values in the cell cultures to which *C. baytopiorum* leaf extract was applied, **A:** C-4 I cell. **B:** Vero cell.

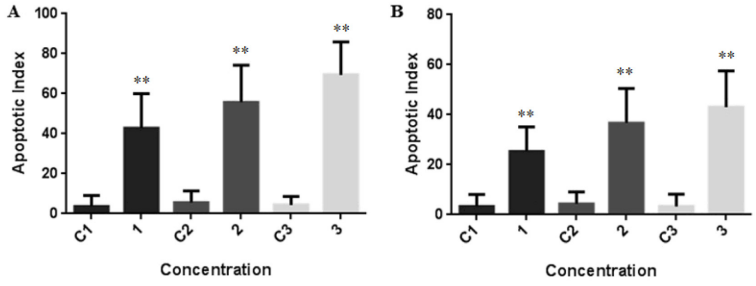

Figure 2. Apoptotic index values in IC50 concentration of *C. baytopiorum* leaf extract at 24th, 48th, and 72nd h. **A:** C-4 I; **B:**Vero (C1:control, 1:experimental group, 24 h. C2: control, 2:experimental group, 48 h. C3:control, 3:experimental group, 72 h). **p<0.01

The extract was applied at the 20th h. Afterwards, they were left for incubation for 72 h. During this period, the measurements were taken with the xCELLigence system. The cell index value (Figure 1) and IC_{40} concentration and its duration were identified based on the obtained graphic and data.

The IC_{50} values of the *C. baytopiorum* plant extract were determined to be 0.01 mg/ml for C-4 I and 0.04 mg/ml for Vero. Cell proliferation was observed 24 h after the application of the plant extracts to Vero cell lines and it was shown that the cells had not proliferated in C-4 I cells.

Selectivity index (SI)

The SI value of the experimental group where the IC_{50} concentration of the *C. baytopiorum* plant extract was applied to the C-4 I cell line was found to be 4.

Apoptotic index

The apoptotic index (AI) values observed in C-4 I cells at the 24th, 48th and 72nd h were respectively 3.6%, 3.3% and 3.3% for the control group and 42.8%, 55.7% and 69.3% for the experimental groups to which the *C. baytopiorum* plant extract was applied (Table 2).

The graphic drawn in accordance with the AI values for all experiment groups is shown in Figure 2. As a result of the statistical analyses made, it was determined that there had been a significant difference between all control and experimental groups ($p<0.05$) (Table 2).

Caspase activation analysis

Caspase 3,7 and 9 activities were measured in C-4 I cells to which the IC_{50} concentration of the *C. baytopiorum* plant extract was applied for 24 h.

The fluorescent values of caspase 3,7 activity of C-4 I cells for the control group were measured to be 207.5 at the 0 min; 197 at the 15th min; 194 at the 20th min; 191 at the 45th min; 187 at the 60th min; 185.5 at the 75th min and 182 at the 90th min. For the experimental group, on the other hand, they were measured to be 303.5 at the 0 min; 291 at the 15th min; 285.5 at the 30th min; 279 at the 45th min; 274 at the 60th min; 270 at the 75th min and 265.5 at the 90th min. The fluorescent values of caspase 9 activity of C-4 I cells for the control group were measured to be 106.5 at the 0 min; 97.5 at the 15th min; 95.5 at the 30th min; 92.5 at the 45th min; 90.5 at the 60th min; 88 at the 75th min and 86.5 at the 90th min. For the experimental group, on the other hand, they were measured to be 117 at the 0 min; 114.5 at the 15th min; 112.5 at the 30th min; 109 at the 45th min; 104 at the 60th min; 100.5 at the 75th min and 99 at the 90th min (Table 3).

Table 2. AI values in the cells to which *C. baytopiorum* leaf extract was applied at the 24th, 48th and 72nd h

Concentration of C.baytopiorum (mg/ml)	*Apoptotic index %*			
	Cells	*24 h*	*48 h*	*72h*
0 (control)	Vero	3.32	3.6	3.39
0.04	Vero	25.36**	36.64**	43.05**
0 (control)	C-4 I	3.67	3.31	3.36
0.01	C-4 I	42.81**	55.72**	69.35**

Significance in comparison with the control group: **p<0.01

Table 3. Fluorescence values of caspases 3, 7 and 9 in C-4 I cells to which IC50 concentration (0.01 mg/ml) of *C. baytopiorum* leaf extract is applied for 24 hours (±SD)

Time (min)	*Activity of Caspase 3,7*		*Activity of Caspase 9*	
	Control	*Experimental Group*	*Control*	*Experimental Group*
0	207,5±1,5	303,5±0,5**	106,5±1,5	117±1*
15	197±1	291±0**	97,5±3,5	114,5±1,5*
30	194±2	285,5±1,5**	95,5±2,5	112,5±0,5*
45	191±0,5	279±1**	92,5±2,5	109±1*
60	187±4	274±1**	90,5±1,5	104±1*
75	185,5±4	270±1**	88±0	100,5±0,5**
90	182±2,5	265,5±0,5**	86,5±0,5	99±1**

Significance in comparison with the control group: *p<0.05, **p<0.01

A significant difference between the experimental group and control group was revealed for which the caspase 3 and 7 activity was examined ($p<0.05$; Table 3).

Real time polymerase chain reaction (qRT-PCR)

The studies were started with 41 gene pairs. FC analysis was performed and fold changes greater than 1.5 were stated with up and down regulation for each gene. The changes in FC and mRNA expression levels in the C-4 I cells to which the plant extract was applied are displayed (Figures 3,4). The FC values were calculated as the analysis group (the experimental group to which the plant extract was applied at the concentration of 0.01 mg/ml) / control group.

As a result of the FC analysis, it was concluded that the expression of 18 genes had been upwardly regulated in comparison with the control group and that the expression of 3 genes had been downstream regulated in comparison with the control group.

It was further determined that the expression level of caspase 3 (1.51) being the caspase mem-

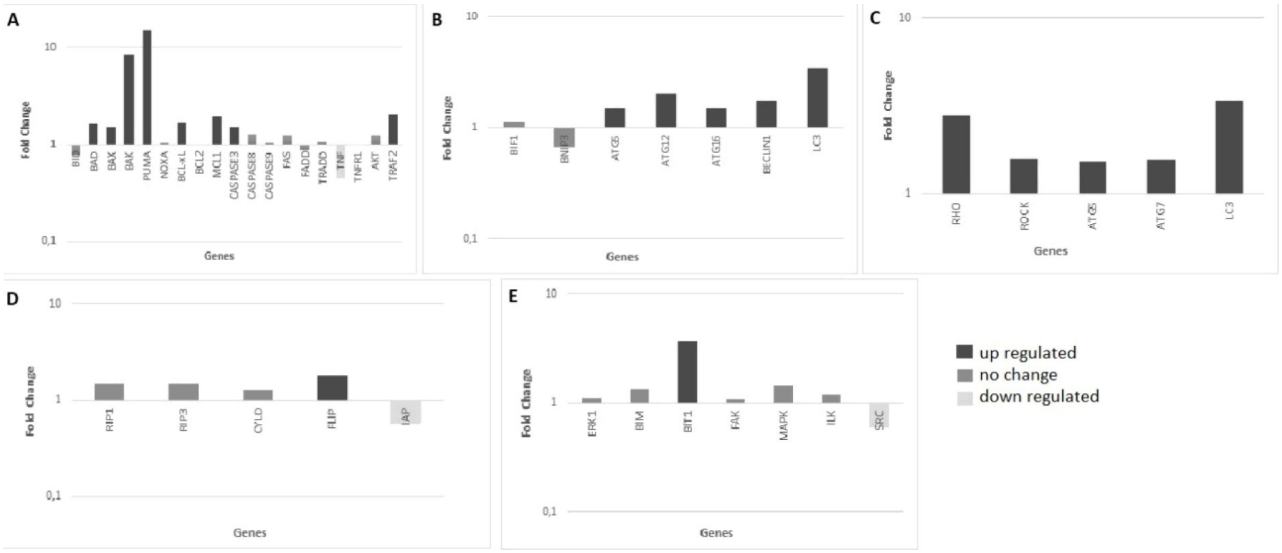

Figure 3. Fold change in the expression of genes associated with **(A)** apoptosis, **(B)** autophagia, **(C)** entosis, **(D)** necroptosis, **(E)** anoikis in the gene expression of C-4 I cells to which *C. baytopiorum* leaf extract is applied.

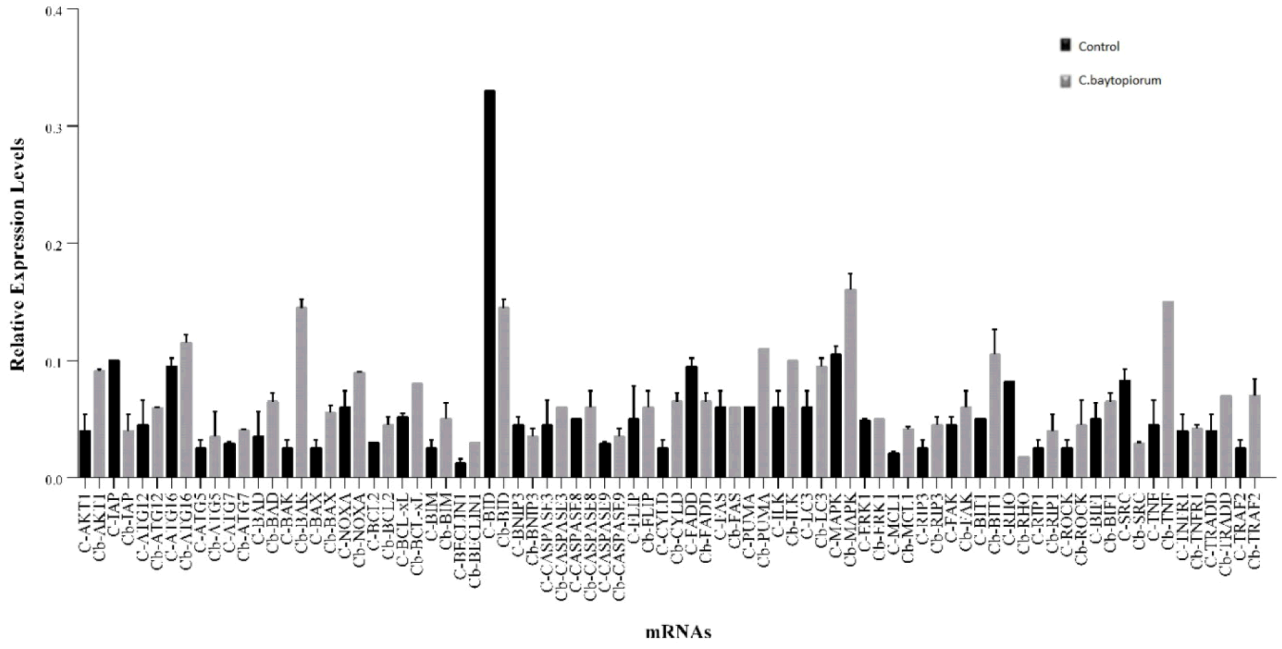

Figure 3. Expression levels of regulators of apoptosis, autophagy, entosis, anoikis, necroptosis cell death pathway in control and C.baytopiorum leaf extract treatment groups.

ber of proapoptotic bad (1.64), bax (1.5), bak (8.55), Puma (15.12) genes and antiapoptotic bcl-xL (1,65), mcl-1 (1.97) genes and TRAF 2 (2.05) being another gene in charge of apoptosis had increased ≥1.5 times in comparison with the control group. The following down regulation was observed in TNFα (-2.22) gene (Figure 4A).

A significant change was not observed in the expression of bid, Noxa (proapoptotic genes), bcl-2 (antiapoptotic) genes belonging to the Bcl-2 gene family, caspase8 and caspase 9 being the caspase members, FAS, FADD, TRADD, TNFR1 that are in charge at the external pathway of apoptosis and Akt being another gene playing a role in apoptosis (Figure 4A).

It was determined that the expression level of Atg5 (1.51), Atg12 (2.03), Atg16 (1.5), Beclin1 (1.74) and LC3 (3.4) genes playing a role in autophagic cell death had increased ≥1.5 times in comparison with the control group. No significant change was observed in the expression of bif-1 and BNIP3 (Figure 4B).

It was found out that the expression level of Rho (2.78), ROCK (1.58), Atg5 (1.51), Atg7 (1.57), LC3 (3.4) genes playing a role in entosis had significantly increased in the extract treatment group (Figure 4C).

The RNA level of the genes playing a role in necroptosis was determined. In this context, it was determined that the expression level of the FLIP (1.76) gene were significantly increased in the extract treatment group in comparison with the control group. It was further determined that the IAP (-1.72) expression had been down-stream regulated. A significant change was not observed in the expression of RIP1, RIP3 and CYLD (Figure 4D).

Moreover, the change in the expressions of the genes associated with anoikis was examined. It was identified that the expression level of the bit1 (3.72) gene had were significantly increased in the extract treatment group. It was also identified that the expression of Src kinase (-1.69) had been down-stream regulated. A significant change was not observed in the expression of ERK1, bim, FAK, MAPK and ILK (Figure 4E).

Discussion

The extracts obtained from the *C. baytopiorum* plants used in our study contain antioxidants (flavonoids, tannins, phenolics), colchicine and its derivatives [2]. In some cases, it was determined that the extracts containing various bioactive compounds had been more effective than a single compound due to their synergistic and antagonistic properties [29-31].

No study with respect to the cytotoxic effects of the methanol extract obtained from the leaf of the *C. baytopiorum* plant on C-4 I cells (cancer cell) and Vero (normal cell) cells as well as the types of cell death it caused, and their molecular structures has been found in the literature.

It is reported that the most important strategy in the development of a medicine for cancer treatment is that normal cells should be damaged less due to the cytotoxic effects of chemotherapy [32] and the stipulation of cell deaths such as apoptosis [33], entosis [34], autophagy [35], anoikis [36,37] and necroptosis [38] plays an important role in cancer cells. The research of anticancer effects of natural products, the majority of which are made of plant extracts and/or compounds, involves research on animals and the determination of cytotoxic effects on cancer cell lines and the effects on the types of cell death prior to clinical studies [39,40].

The cytotoxic effect of 6 different concentrations of the plant extract (1: 2.5, 2: 1, 3: 0.25, 4: 0.1, 5: 0.025, 6:0.01 mg/ml) applied on the cells within the scope of our study was determined with the xCELLingence RTCA device. The IC_{50} concentrations resulting from the application of the methanol extract obtained from the leaf of the *C. baytopiorum* plant were determined to be 0.04 mg/ml and 0.01 mg/ml, respectively. A graphic was obtained after the plant extracts were applied to the cells at different concentrations (Figure 1). According to the obtained graphic, it was determined that the 0.01 mg/ml and 0.25 mg/ml concentrations of the extract of *C. baytopiorum* had doubled the number of cells 20 h after the application to Vero cells (normal cell) and proliferated the cells (Figure 1). All concentrations showed a cytotoxic effect after the application of the plant extract to C-4 I cells (cancer cell) and did not result in proliferation. It was identified that the cytotoxic effect of a 0.01 mg/ml concentration (IC_{50} value) of the *C. baytopiorum* extract with a greater cytotoxic effect on the cancer cell had been less on normal cells.

The rate of IC_{50} values of the substance applied to cancer and normal cell lines to each other (SI) is an important pharmaceutical parameter for the possibility of its usage in the clinic in the future. If the SI value is >2, and this shows that it is an authentic anticancer substance and if it is >3, this means that it displays a higher selectivity [41]. For this purpose, the extract used in our study was applied to cancer (C-4 I) and normal (Vero) cell lines and the SI values were determined. According to the obtained findings, it was concluded that the extract of *C. baytopiorum* plant had displayed a high selective toxicity (SI = 4). In this sense, the potential of the extract of *C. baytopiorum* plant from

natural products to be used as an anticarcinogenic agent in C-4 I cells has been revealed in our *in vitro* study.

The SI value of Actinomycin D being an anticancer agent applied to HeLa and Vero cells in a study was determined. The SI value of Actinomycin D was found to be 13.5 and it was shown that it had a high selective toxic effect [42].

As a result of examination under a fluorescent microscope for both C-4 I and Vero cell lines the groups in which the IC_{50} concentration was applied for 24, 48 and 72 h, the apoptic index of the extracts of *C. baytopiorum* increased more in cancer cells in comparison with normal cells depending on time (Figure 2).

In a study, the methanol extracts prepared with the tubers of *Colchicum umbrosum* and *Colchicum baytopiorum* plants were applied to HeLa cell lines at the concentrations of 0.001, 0.05, 0.1, 0.5 and 1 mg/ml and the apoptotic index values were determined [43]. It was determined that the highest apoptotic index value of the experiment group in which 0.1 mg/ml concentration of *C. baytopiorum* extract had been at the 48th h. The results obtained in our study are coherent with this and other similar research.

The determination of the apoptotic index in malignant growth is accepted as a prognostic indicator [44].

As a result of the assessment made in consideration of cytotoxicity for 24 h, as well as the SI and AI values for the *C. baytopiorum* extract within the scope of this study, it was determined that the most effective concentration for C-4 I cells had been 0.01 mg/ml and molecular research involving the activation of caspases 3, 7 and 9 and qRT-PCR analyses was performed for this purpose. As a result of the analyses of caspases 3,7 and 9, a significant increase ($p<0.05$) was observed in caspase activation in comparison with the control group (Table 3).

In the study, the activation of caspases 3,7 and 9 with an important role in apoptosis pathways in terms of the apoptosis caused by hypobaric stress conditions on the MCF-7 cell line was identified. According to the findings, caspase 3,7 and caspase 9 kits were applied separately to the cell lines and the absorbance values were determined with a specfluorometer at the wavelength of 490-520 mm. It was also shown that there had been a time-related significant difference between the experimental group whose caspase 3,7 activation values were determined and the experimental group whose caspase 9 activation values were determined [45].

In order to enhance the effectiveness of a cancer treatment, it is important to review the genes associated with the cell death types of apoptosis, autophagy, entosis, necroptosis and anoikis, assess the molecular mechanisms related thereto and develop new strategies that will improve the antitumor effect on the target organs. In this context, relative expression levels of the genes associated with the cell death types as a result of the application of 0.01 mg/ml concentration of *C. baytopiorum* extract in C-4 I cell lines for 24 h were determined with the qRT-PCR in our study (Figure 3).

In this study, it was determined that the expression of the proapoptotic bid being a member of the Bcl-2 gene family associated with apoptosis, Noxa and antiapoptotic bcl-2 genes, caspase 8 and caspase 9, FAS, FADD, TRADD, TNFR1 in charge at the external pathway of Akt and apoptosis with a receptor agent had not displayed a significant change in comparison with the control group. It was identified that the expression of bad (1.64), bax (1.5), bak (8.55), Puma (15.12), bcl-xL (1.65), mcl-1 (1.97) genes being a member of Bcl-2 gene family in charge of the internal pathway of apoptosis had increased in comparison with the control group. Moreover, it was detected that there had been a significant increase (FC ≥ 1.5) in the expression level of caspase 3 (1.51) and TRAF2 (2.05) being another gene in charge of apoptosis. The following regulation was observed in the TNFα (-2.22) gene playing a role in the external pathway of apoptosis with a receptor agent (Figure 4a).

In a study [46], TNF-α induced cytotoxicity was increased with p38MAPK inhibition in rodent fibroblasts. However, LNCaP was shown to provide protection against TNF-α induced apoptosis of p38MAPK in LNCaP prostate cancer cells. Additionally, very limited information is available about p38 MAPK signaling playing a role in TNF-α induced apoptosis in glioma cells. In that study, the hypothesis suggesting that p38MAPK activation mediated the apoptosis induced with TNF-α in glioma C6 cells makes us consider p38MAPK as a potential target for glioma treatment.

The determination that caspase 3 increased with qRT-PCR is supported by the analysis results of caspase 3 activation. According to the obtained findings, it was shown that a 0.01 mg/ml concentration of *C. baytopiorum* extract had increased the expression of the members of the proapoptotic Bcl-2 gene family (bad, bax, bak, Puma) at the C-4 I cell line and triggered the mitochondrial pathway. Furthermore, the increase in caspase 3 playing a role in the occurrence of apoptosis mediating cytochrome c oscillation from mitochondrion to cytoplasma is in parallel with this finding.

It was shown that caspase 3 treatment could induce a common apoptosis in addition to etopo-

side treatment and led to the decrease of tumour volume in an AH130 liver tumour model.

Apoptosis is triggered with the activation of p53, being a tumour repressing protein, that induces the transcription of DR5, bax, bak, Noxa, Puma as a result of damage occurring in the DNA and inhibits the transcription of antiapoptotic genes like Bcl-2.

Most tumours (about 90%) have such mutations that deactivate p53 protein, repress its expression and prevent Puma from triggering apoptosis [47]. In this context, Puma is an important target molecule in cancer treatment.

In a study conducted by Aubrey et al in 2017, it was reported that overexpression of pre-apoptosis antiapoptotic Bcl-2 gene had prevented gamma-radiation induced death of many varieties of leukocyte populations [48]. This prevents the need to take action against excessive reproduction of hematopoietic stem cells in bone marrow, which are considered to be cancer stem cells in this model at the beginning of lymphoma/leukaemia. This concept can also be applied to epithelial cancer types. The underlying reason is that excessive expression of antiapoptotic Bcl-2 delays the development of liver cancer and in contrast the loss of antiapoptotic Mcl 1 supports its development. According to these findings, the fact that a significant increase of Mcl 1 prevents the development of cancer despite the increase of proapoptotic genes is supported in our study.

The cytoplasmic interaction of p53 with proteins being members of the antiapoptotic Bcl-2 gene family in mitochondrion causes the release of apoptotic factors like cytochrome c from the mitochondrial external membrane. Furthermore, p53 can directly interact with bak and/or bax and activate the change of the permeability of the external mitochondrial membrane [49].

Pharmacological applications might lead to cell death by causing stress in endoplasmic reticulum (ER). As a result of ER stress, a TRAF2 increase playing a role in external pathways with receptor mediation is observed. A TRAF2 increase results in down-regulated JNK activation. JNK is phosphorylated and ensures that only the proapoptotic proteins containing BH-3 regions activate. This causes bax and bak oligomerization and enables the permeability of the external mitochondrial membrane and the mitochondrial apoptotic process is managed [50]. The reason why TRAF2 increased as a result of the application of a 0.01 mg/ml concentration of *C. baytopiorum* extract to C-4 I cell lines in comparison with the control group shows that such an increase occurs in this situation. The increase of proapoptotic bax and bak in charge of the mitochondrial internal pathway of apoptosis also supports this finding.

It was also investigated in our study as to whether the extract of *C. baytopiorum* plant triggers autophagic cell death in C-4 I cells. It was detected that the expression levels of Atg5 (1.51), Atg12 (2.03), Atg16 (1.5), Beclin1 (1.74) and LC3 (3.4) genes associated with autophagic cell death had obviously increased after the application of the extract (Figure 4B).

Beclin-1 plays a central role in the formation of the autophagosome structure and in the cooperation between autophagy and apoptosis [51]. This function of Beclin-1 is regulated by caspases. It is divided into Beclin 1, Beclic-1-C and Beclin-1-N by caspase 3,7 and caspase 8 and autophagy is initiated. At the same time, the translocation of Beclin1-C to mitochondrion is achieved and apoptosis is triggered [29,52].

In this study, the increase of bcl-xL, being an antiapoptotic, is associated with autophalgy. bcl-xL plays an important role between apoptosis and autophagy [53,54].

It was shown that apoptosis and autophagy had been activated together in human colon cancer cells to which oxaliplatin and bortezomib were applied as chemotherapeutic agents by Kim et. Al in 2014 [54]. Separating from Bcl-xL during the application of oxaliplatin and bortezomib, Beclin1 causes initiation of autophagy. According to these results, the combined application of oxaliplatin and bortezomib inhibits the growth of tumours by Beclin1/bcl-xL. This study of ours supports the increase of Beclin1, being a gene associated with autophagy and other similar studies.

Recent studies suggest that Atg 5 and Atg 12 proteins also establish a relationship between autophagy and apoptosis along with Beclin1.

In another study of ours, a 0.1 mg/ml concentration of the methanol extract prepared with the tubers of the *Colcicum baytopiorum* plant was applied to HeLa cells and its effect on autophagic and apoptotic cell death was investigated. Gene expressions of Bif, Beclin1, BNIP-3, Atg 12, Atg 5, DAPK, bcl-xL, bad, bid, Puma, Noxa, Fas, TNFR1, caspase 3, caspase 8 and caspase 9 were reviewed. It was observed that an increase had occurred in Bcl-xL, BNIP-3, Bif-1, Beclin-1, Atg12 and Atg5 genes which played a role in autophagy and apoptosis and it was detected that autophagic cell death had also been triggered along with apoptosis [31]. These findings support the results obtained in this study.

New and effective treatment models are suggested in various studies for the elimination of cancer cells where apoptotic and autophagic cell death

types are stimulated together with different agents. For example, Lockshin and Zakari (2007) showed in their study that the application of etoposide had an increasing effect with apoptosis on rats' embryonic fibroblast cells (MEF) and the application of ceramide in breast and colon carcinomas had activated autophagy simultaneously with apoptosis [55].

Ag12-Atg5-Atg16 form a triple complex in autophagy and expands the vesicle. The formation of this complex results from the activation of Atg 7. The phagophore is extended and closed with Atg5-Atg12 and LC3 two quasi-ubiquitin systems. LC3 is an important indicator for the determination of phagophore and autophagosomes [56]. In our study, it was shown that there had been an increase in the expression of Atg 7 (1.57) protein (Figure 4B). Moreover, it was detected that LC3 [3.4] which played a role in the formation of autophagosome in C-4 I cells to which the plant extract was applied had also obviously increased. Atg 5, Atg 7 and LC3 plays a role in entosis as well.

An increase was observed in the expressions of Rho (2.78) and ROCK (1.58) that were effective in entosis within the scope of this study (Figure 4C). Rho and its effector ROCK also play a role in in-cell signaling. Activated ROCK triggers the phosphorylation of the slight myosin chain. This is effective in apoptotic processes such as membrane budding, cell fractionation and phagocytosis of apoptotic objects [57].

The findings must be supported with morphological imaging techniques in order to certainly claim the existence of entosis, although an increase was detected in the expression levels of Rho, ROCK, Atg5, Atg7 and LC3 genes associated with entosis.

On the other hand, there has been some research where the relationship between apoptosis and anoikis was revealed. It was shown in some studies that lanthanum, being one of the rare earth elements, had encouraged apoptosis in some cancer cells. Yamaki et. al (2007) used HeLa cells of cervix cancer origin in their study, which is a cell line widely used in experiments [58]. The role of RhoG in anoikis was investigated in that study with the use of HeLa cells that lack RhoG. It was observed that lack of RhoG had encouraged apoptosis in HeLa cell cultures and PI3K activity had been necessary for the RhoG associated antiapoptotic effect. ELMO and Dock180 were seen to be unnecessary for the repression of RhoG associated PI3K/Akt signaling pathway and anoikis.

The signal mechanisms inhibiting anoikis were investigated with experimental mechanisms. For instance, it was shown in a study that Ras activation in MCF-10A human breast epithelial cells had blocked anoikis [17].

In this study, the changes in the expressions of bit1, Src kinase, ERK1, bim, FAK, MAPK and ILK genes associated with anoikis were examined and it was detected that an increase had occurred in bit1 expression playing a role in apoptosis and that the Src kinase had decreased. It was further determined that the expression of ERK1, bim, FAK, MAPK and ILK genes had not undergone a change in comparison with the control group (Figure 4E). According to the obtained findings, a 0.01 mg/ml concentration of *C. baytopiorum* extract did not cause anoikis in C-4 I cells.

It was also investigated whether the plant extract used had caused necroptosis, being one of the death types in C-4 I cell lines. It was detected that the IAP (-1.72) expression had decreased and RIP1, RIP3 and CYLD expression had remained unchanged in comparison with the control group (Figure 4D). The fact that there was no increase or change in gene expressions indicates that necroptosis had not occurred. A decrease in IAP expression, which played a role in the repression of apoptosis, supports the occurrence of apoptosis.

Down-regulated expression of the molecules that mediate necroptosis in cancer is shown. It was determined as a result of a study that the expressions of the molecules with RIP1, RIP3, MLKL and CYLD content playing a role in necroptosis in various types of cancer had decreased or a mutation had occurred in these molecules. In another study in which more than 60 cancer cell lines were used, it was concluded that about 23 of them had not had the RIPK3 protein. It was also identified that the expression of RIP3 and CYLD had obviously decreased in chronic lymphocytic leukaemia. The expression of RIP1 and RIP3 significantly decreased in primary tumours that were compared with colon, breast, lung cancer cells and suitable normal tissues [59-61].

Consequently, it was determined that the methanol extract obtained from the leaf of *C. baytopiorum* plant had a high selective toxic effect in accordance with the parameters subjected to an assessment. The extract of C.baytopiorum was determined to have less toxicity effect on normal cells (Vero) in comparison with the cancer cell line (C-4 I). Besides, it was identified that the most effective concentration of the extract of *C. baytopiorum* that was 0.01 mg/ml had triggered the internal apoptotic pathway in C-4 I cells by causing an increase in the expression level of bad, bax, bak, Puma genes. Furthermore, it was detected that autophagy had also stimulated the increase in the expressions of At5, Atg12, Atg16'nın and Beclin1, LC3 genes associated with autophagy. In this context, it was revealed that it had showed its anti-tumor effect

by triggering autophagic cell death along with apoptosis and that this cooperation was related to Beclin-1 and bcl-xL interaction.

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Conflict of interests

The authors declare no conflict of interests.

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