Chapter

In Vitro Cytotoxic and Apoptosis Induction Potential of Two Plant Extracts on HeLa Cells

Özlem Dağdeviren Özsöylemez and Gül Özcan

Abstract

Natural products are commonly used for developing anticancer drugs that are beneficial for various cancer types. The aim of this study is to apply *Colchicum umbrosum* Steven and *Colchicum baytopiorum* CD Brickell (one of the endemic species in Turkey) extracts on HeLa cell lines and determine changes in cytotoxicity and viability. For this aim, kinetic parameters such as proliferation rate have been determined by MTT assay, and apoptotic index (AI) has been researched by fluorescence microscopies using DAPI staining. Also, some apoptosis-related genes have been examined by the RT-PCR method. Five different concentrations of both extracts from the two Colchicum species have cytotoxic effects and it has been understood that HeLa cells were more sensitive to the most effective concentration of the *C. baytopiorum* extract, which is 0.1 mg/ml, and it showed antitumor effects by causing apoptosis for 48 h. The cytotoxic activity and apoptotic effects of *Colchicum umbrosum* Steven and *Colchicum baytopiorum* (Colchicaceae/Liliaceae) have been studied for the first time on HeLa cell lines. We suggested that the medicines derived from natural products seem to be a new promising treatment for cancer.

Keywords: apoptosis, *Bcl-2* gene family, cancer, *Colchicum baytopiorum*, *Colchicum umbrosum*, HeLa cells

1. Introduction

Cancer is a disease characterized by constant clonal proliferation of the somatic cells due to which the homeostatic feedback mechanism gets out of control through genetic and epigenetic alterations and kills by invading normal tissues [1–4]. Natural products extracted from plants, microbes, and other marine organisms have secondary or non-essential metabolism. Natural products have gained importance in novel anticancer agents' discovery and investigation of more effective drugs [5, 6]. Colchicine, which is obtained from plants of the genus Colchicum belonging to Liliaceae (Colchicaceae) family, is one of the natural product. Colchicine is an anti-neoplastic drug used in cancer therapy as a mitotic inhibitor [7].

Apoptosis plays a critical role in the elimination of damaged or undesirable cells and the development and maintenance of homeostasis. Disturbances in these

processes cause various diseases ranging from cancer and autoimmune disorders to ischemic injuries and neurodegenerative diseases [8–14].

Apoptosis is encouraged by cell signaling, lack of growth factors, the release of granzyme, specific receptor-ligand interactions, and various stress factors (chemotherapy, radiation, etc.). Then, the cell death process is initiated. Some of the original proteins play a role in this process. As a consequence thereof, signals are transferred to the execution mechanism with the help of adaptor proteins and are regulated the mitochondrial permeability by bcl-2 family proteins. An increase in permeability of the outer mitochondrial membrane is caused by the release of cytochrome c. Cytochrome c is connected to apoptosis protease activation factor (Apaf-1) in the cytosol. Apaf-1 binds to the pro-caspase-9 molecule by oligomerization in the presence of adenosine triphosphate (ATP) and constitutes apoptosome. The activated caspase-9 plays a role in the activation of caspase-3 and caspase-7. Nevertheless, the *bcl-2* in the mitochondrial membrane prevents activation of caspases by preventing the increase of mitochondrial permeability and making stable Apaf-1 and so on proteins. Thus, apoptosis does not occur. *bcl-xL,* which is a member of the bcl-2 family, and *bcl-2* play the anti-apoptotic role. *bax*, *bad,* and *bak* are triggered by apoptosis [15–22].

In recent years, investigating the molecular mechanisms of apoptosis by using agents has become extremely important in cancer treatment [23–26]. Our study is aimed to investigate the cytotoxic effects of different concentrations of two extracts that belong to the genus *Colchicum*, one of them is endemic in Turkey (*Colchicum umbrosum* Steven (S.) and *Colchicum baytopiorum* CD Brickell (CD) is an endemic species in Turkey [27, 28], on HeLa cells and also show the molecules that play a role in the regulation of molecular mechanisms of the resultant apoptosis.

2. Materials and methods

2.1 Cell line and culture conditions

Human cervical cancer cell line (HeLa) cells were obtained from American Type Culture Collection, HeLa (CCL-2). The cells were cultured in a 25-cm^2 flask following Minimum Essential Medium (Sigma, MEM) containing 10% FBS (Gibco Lab.), penicillin (100 unit/ml), and streptomycin (50 mg/ml). The cells were incubated at 37°C in a humidified %5 CO2 incubator [29].

2.2 Plant material and extraction

Corms of *Colchicum baytopiorum* (ISTE: 81438, Antalya-Termessos, July 5, 2005) and *Colchicum umbrosum* (ISTE: 85333, Bolu-Abant, June 25, 2008) were dried. All dried plant materials were extracted with methanol. Methanol extracts were evaporated in a rotavapor. Plant extracts were prepared in five different concentrations (0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml) in MEM, supplemented with %10 FBS. Prepared concentrations were treated to HeLa cells in time period of 24, 48, and 72 h [30, 31].

2.3 Assays for in vitro cytotoxicity

Cytotoxic effects of plant extracts were measured by using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma) assay. Cells were seeded onto a 96 well plate (30,000 cells/well), allowed to attach overnight, and then exposed to five different concentrations of plant extracts for 24, 48, and 72 h. MTT solution dissolved in PBS (40 μl/well) was added. Cells were incubated at 37°C for 4 h. Formazan crystals were dissolved in 160 μl DMSO (dimethyl sulfoxide) at 37°C for 1 h. The plate was read on ELISA reader at 570 nm reference of 690 nm (μQuant, Bio-tek) [32].

2.4 Apoptotic Index

Apoptotic cells showed characteristic fragmented nuclei, whereas survivors showed an intact nuclear morphology. These cells were fixed with cold methanol. The cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) nuclear staining mounting solution for the determination of the apoptotic index (AI) [33]. Then, the slides were washed in phosphate-buffered saline (PBS). The AI was calculated by scoring the percentage of apoptotic cells in control and the extract treatment (0.1 mg/ml) group of 1000 cells and at least 30 areas/each slide under the fluorescence microscope [34].

2.5 Phase-contrast microscopies

After the treatment with C. baytopiorum extract (0.1mg/ml), the morphological changes in HeLa cells were examined after 24, 48, and 72 h under phase contrast microscopies (x200).

2.6 Light microscopies

The morphological changes that occurred in HeLa cells were examined with Giemsa stain and Feulgen method under a light microscope (×1000) after 24, 48, and 72 h [35].

2.7 The isolation of total RNA

Total RNA was isolated from cells in the control and the extract treatment (0.1 mg/ml) group, for 48 h by using a total RNA isolation kit (Invitrogen PureLink Micro-to-Midi Total RNA Isolation Kit, Cat. No: 12183-180). For the determination of the amount of isolated total RNA (diluted 1: 200), spectrophotometer (Cintra 20, GBC) was used at 260 nm [36].

2.8 Reverse transcriptase (RT)-PCR

A total of 21 μl RNase-free water was put in each PCR tube. In this mix, 25 μl of the 2x Reaction mix (0.4 mM MSO_4 of each deoxynucleoside triphosphate) and 2 l of RT/Platinum ™ Taq Max were added. Then, 1l of *bcl-x, bik, mcl-1, bfl-1*, and β-*actin* (Takara BCL-2 familly, Cat no.6623) and 1l of total RNA of each experimental group was added to the tube for one-step RT-PCR (The Super Script™ One-Step RT-PCR Kit Invitrogen, Cat no. 10928-042).

Reverse transcription and amplification were performed in TC412 Techne. The RT-PCR profile used was as follows: a reverse transcription stage at 55°C for 30 min, followed by an initial denaturation stage at 94°C for 2 min. This was then followed by 40 amplification cycles at 94°C for 15 s, at 60°C for 30 s, and at 68°C for 1 min, and a final extension step at 68°C for 5 min. The resulting PCR products were visualized by electrophoresis through 1.8% agarose gel containing ethidium bromide and then visualized under UV light [37].

2.9 Statistical analysis

Apoptotic index and cytotoxicity were analyzed by one-way ANOVA, followed by Dunnett's test for separate comparisons with the control group and the T-test for separate comparisons with each of the groups. Differences were considered significant at $p < 0.05$ (GraphPad Prism version 4.00, GraphPad Software, San Diego California USA).

3. Results

In our study, the cytotoxicity of all extracts was determined by MTT assay. Accordingly Cytotoxicity calculation method, it has been determined that the cytotoxicity and % vitality (calculated by assuming the control group as 100%) in HeLa cells, treated with the extracts from two species of Colchicum plant, are being increased in concentration compared to the control group ($p < 0.05$). Different concentrations of both extracts from the two Colchicum species have cytotoxic effects ($p < 0.05$) and HeLa cells are more sensitive to the species of *C. baytopiorum*. The meaningful cytotoxic effect of the 0.001 mg/ml concentration extracted from *C. umbrosum* has been occurred in only 72 h, compared to the control group ($p < 0.01$) (**Figures 1** and **2**).

Determined viability% values of HeLa cells treated with *C. baytopiorum* are respectively 70% for B1, 74% for B2, 58% for B3, 75% for B4, 77% for B5 in 24 h; 28% for B1, 23% for B2, 20% for B3, 26% for B4, 20% for B5 in 48 h; 15% for B1, 19% for B2, 27% for B3, 17% for B4, 14% for B5 in 72 h. Vitality % values of HeLa cells treated with *C. umbrosum* are respectively 101% for U1, 70% for U2, 82% for U3, 52% for U4, 77% for U5 in 24 h; 99% for U1, 28% for U2, 19% for U3, 33% for U4, 21% for U5 in 48 h; 63% for U1, 20% for U2, 21% for U3, 20% for U4, 23% for U5 in 72 h.

Apoptotic index is determined by fluorescence microscopy using DAPI staining (**Figure 3**). DAPI staining was also conducted to show the apoptosis of HeLa cells. The AI was calculated as follows:

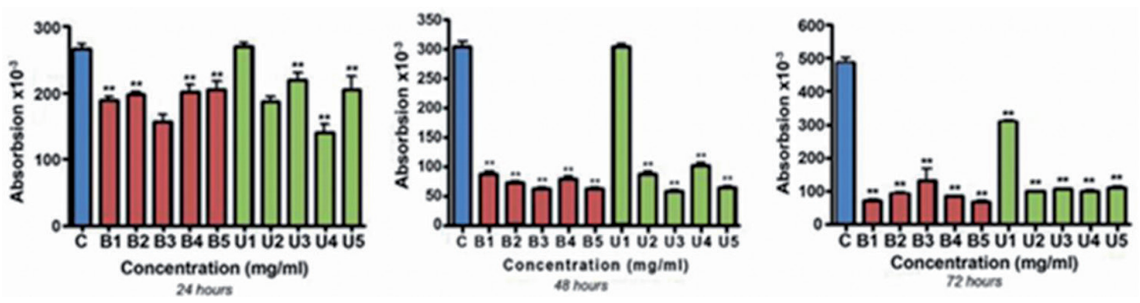

Figure 1.
*MTT results of HeLa cells treated with C. baytopiorum and C. umbrosum (B1 and U1: 0.001, B2 and U2: 0.05, B3 and U3: 0.1, B4 and U4: 0.5, B5 and U5: 1 mg/ml) for 24, 48, and 72 h. *p < 0.05, **p < 0.01 (in comparison to control).*

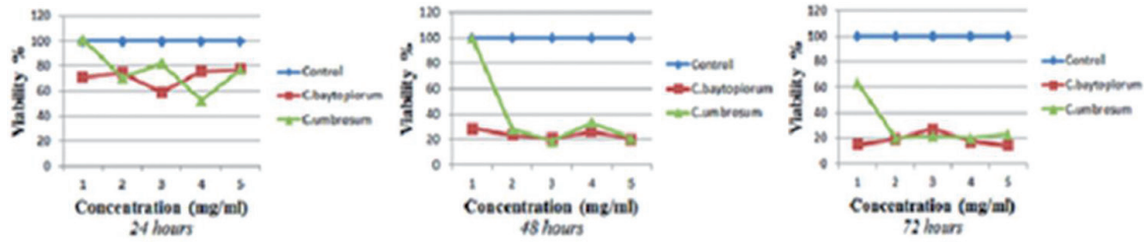

Figure 2.
Viability % values of HeLa cells treated with C. baytopiorum and C. umbrosum (1: 0.001, 2: 0.05, 3: 0.1, 4: 0.5, 5: 1 mg/ml) for 24, 48, and 72 h.

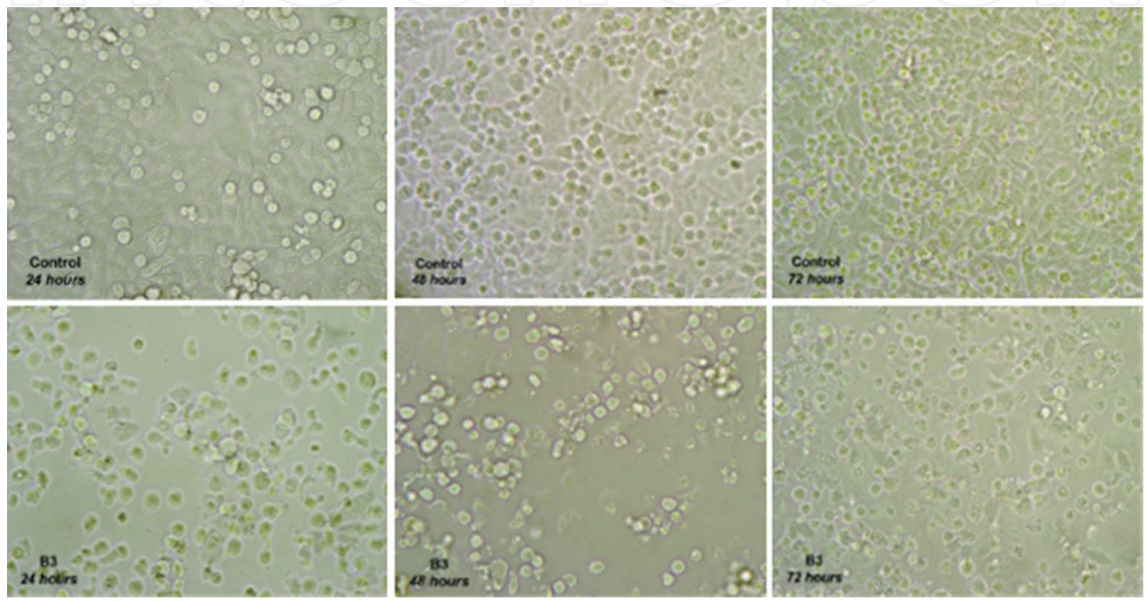

Figure 3.
Phase-contrast microscopy of control and B3 (0.1 mg/ml) concentration of the C. baytopiorum extract treatments groups on HeLa cells for 24, 48, and 72 h (×200).

AI = (number of apoptotic cells/total number counted) × 100%.

Apoptotic index values of HeLa cells treated with *C. baytopiorum* and *C. umbrosum* for 24, 48, and 72 h are shown in **Figure 4**. AI values were determined as 2.3% for control group, 18.3% for B1, 16.4% for B2, 12.37% for B3, 25.12% for B4, 18.02% for B5 in the application of the *C. baytopiorum* extract; 8.66% for U1, 6.78% for U2, 14.49% for U3, 15.88% for U4, 18.91% for U5 in the application of the *C. umbrosum* extract in 24 h.

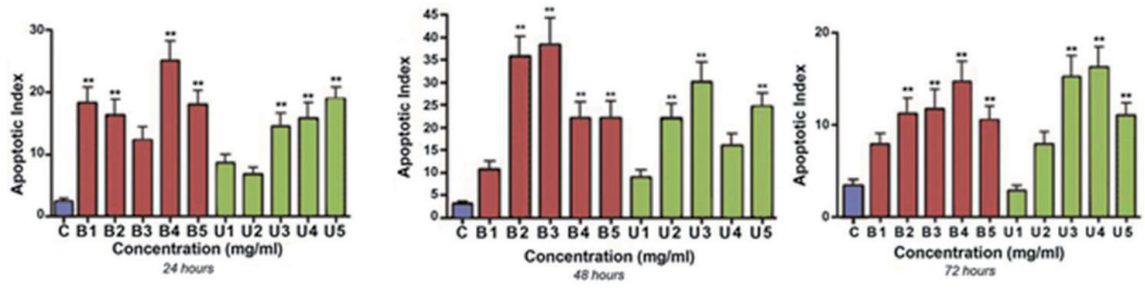

Figure 4.
*Determined apoptotic index (AI) values of HeLa cells treated with C. baytopiorum and C. umbrosum (B1 and U1: 0.001, B2 and U2: 0.05, B3 and U3: 0.1, B4 and U4: 0.5, B5 and U5: 1 mg/ml) for 24, 48, and 72 h. **$p < 0.01$ (in comparison to control).*

In our experiments, in 24 h, it was determined significant cytotoxic effect according to the control ($p < 0.01$) in the experimental groups B1, B2, B4 and B5 applied the extract of *C. baytopiorum* and the experimental groups U3, U4 and U5 applied *C. umbrosum* extract.

AI values were determined as 2.84% for control group, 10.67% for B1, 35.91% for B2, 38.38% for B3, 22.04% for B4, 22.03% for B5 in the application of the C. *baytopiorum* extract; 8.9% for U1, 22.05% for U2, 30.19% for U3, 16.01% for U4, 23.58% for U5 in the application of the *C. umbrosum* extract in 48 h.

In our experiments in B2, B3, B4, and B5 experimental groups applied *C. baytopiorum* extract and U2, U3, U5 experimental groups applied *C. umbrosum*

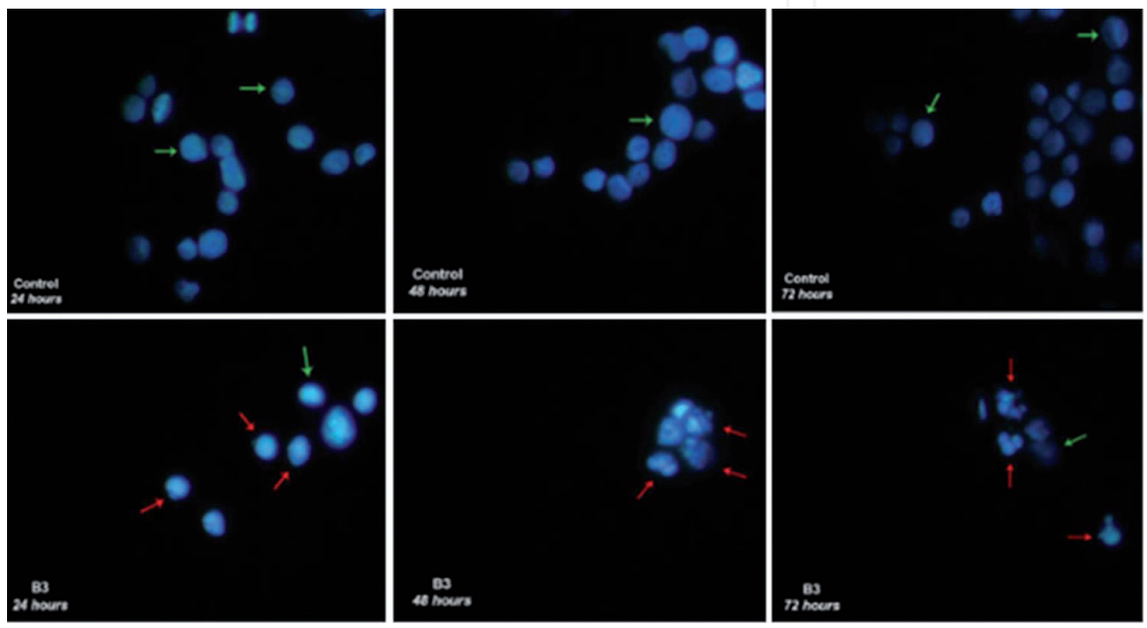

Figure 5.
Fluorescence microscopy of control and B3 (0.1 mg/ml) concentration of the C. baytopiorum extract treatments groups on HeLa cells for 24, 48, and 72 h (×1000; DAPI) (→: non-apoptotic cell, →: apoptotic cell).

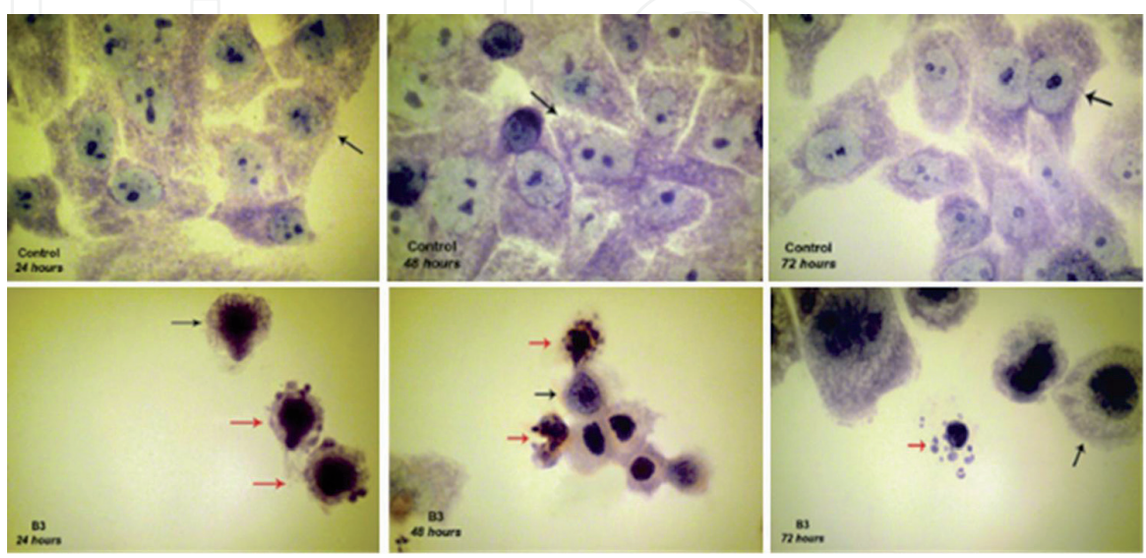

Figure 6.
Light microscopy of control and B3 (0.1 mg/ml) concentration of the C. baytopiorum extract treatments groups on HeLa cells for 24, 48, and 72 h (×1000; Giemsa) (→: non-apoptotic cell, →: apoptotic cell).

extract were determined to have a significant cytotoxic effect according to control ($p < 0.01$) in 48 h.

AI values were determined as 2.92% for control group, 7.89% for B1, 11.19% for B2, 11.76% for B3, 14.72% for B4, 10.5% for B5 in the application of the *C. baytopiorum*

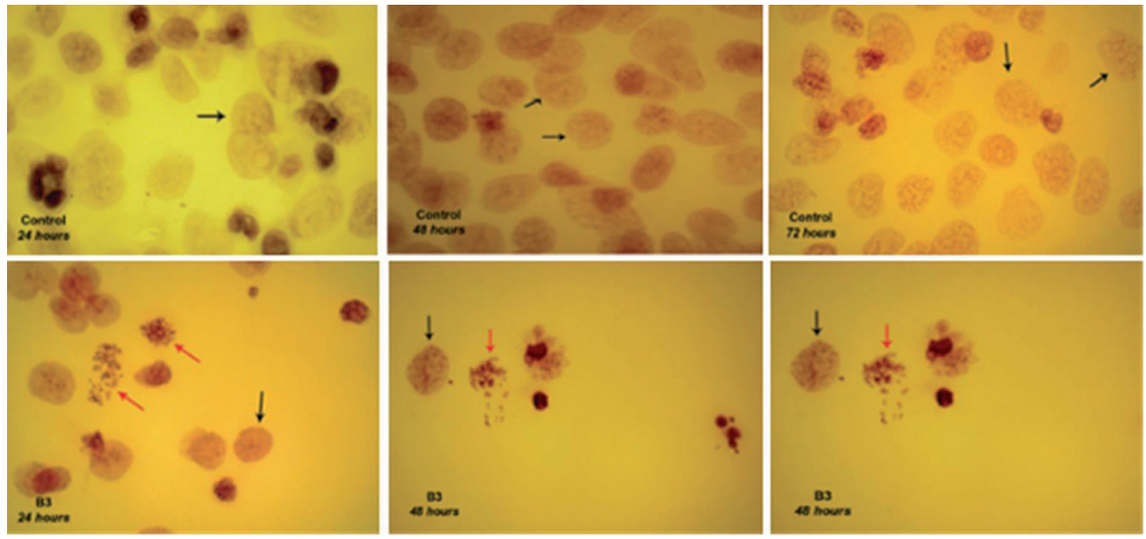

Figure 7.
Light microscopy of control and B3 (0.1 mg/ml) concentration of the C. baytopiorum extract treatments groups on HeLa cells for 24, 48, and 72 h (×1000; Feulgen) (→: non-apoptotic cell, →: apoptotic cell).

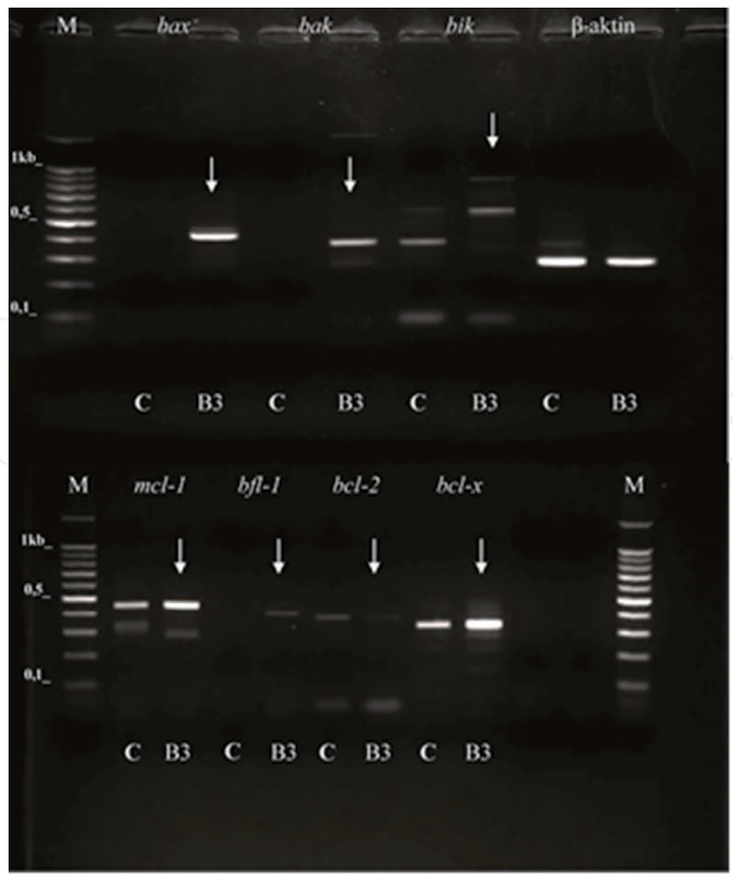

Figure 8.
RT-PCR analysis of bcl-2 members of control and B3 (0.1 mg/ml) concentration of the C. baytopiorum extract treatments groups on HeLa cells for 48 h (M: 1.5 kb marker, C: control, B3: 0.1 mg/ml).

extract; 2.82% for U1, 7.91% for U2, 15.22% for U3, 16.23% for U4, 11% for U5 in the application of the *C. umbrosum* extract in 72 h.

In our experiments, in 72 h, it was determined significant cytotoxic effect according to the control ($p < 0.01$) in the experimental groups B1, B2, B4 and B5 applied the extract of *C. baytopiorum* and the experimental groups U3, U4 and U5 applied *C. umbrosum* extract.

The methanol extract of *C. baytopiorum* has been treated with the most effective concentration of 0.1 mg/ml in 48 h experiment group (after the evaluation of cytotoxicity and AI value).

The morphological changes belonging to apoptosis such as blebbing of membrane and apoptotic body including cell organelles and chromatin parts [32, 33] have been shown through phase-contrast, fluorescence, and light microscopies (**Figures 3** and **5**–**7**).

To determine the apoptosis at the molecular level, the expression rates of the genes of the *bax*, *bak*, *bik*, *mcl-1*, *bfl-1*, *bcl2*, and *bcl-x,* which are members of the *bcl-2* gene family, have been searched through the RT-PCR method. In the 48 h experimental group of HeLa cells which were treated with the concentration of 0.1 mg/ml of *C. baytopiorum* extract, an increase in the expression of the *mcl-1* and *bcl-x* genes by compared to the control group was observed, and an increase of the expression of *bax, bak, bik* and *bfl-1* genes was also determined . In contrast, any expression was observed in the *bcl-2* gene (**Figure 8**).

4. Discussion

Some studies reported that crude extracts of natural products containing a variety of molecules that exhibit antitumoral activity are highly effective in cancer cell death [38–40].

However, some reports suggested that natural product mixtures often claim that they are more effective than purified compounds due to synergistic, additive, or antagonistic interactions [41]. Also, different species of the same genus may have different concentrations of the same compounds [42].

In our study, cytotoxicity of all extracts was determined by MTT assay. Accordingly, it has been determined that the cytotoxicity and % vitality (calculated by assuming the control group as 100%) in HeLa cells, treated with the extracts from two species of Colchicum plant, are being increased in concentration compared to the control group ($p < 0.05$). Different concentrations of both extracts from the two Colchicum species have cytotoxic effects ($p < 0.05$) and HeLa cells are more sensitive to the species of *C. baytopiorum*. The meaningful cytotoxic effect of the 0.001 mg/ml concentration extracted from *C. umbrosum* has been occurred in only 72 h, compared to the control group ($p < 0.01$) (**Figures 1** and **2**).

Determined viability % values of HeLa cells treated with *C. baytopiorum* are, respectively, 70% for B1, 74% for B2, 58% for B3, 75% for B4, 77% for B5 in 24 h; 28% for B1, 23% for B2, 20% for B3, 26% for B4, 20% for B5 in 48 h; and 15% for B1, 19% for B2, 27% for B3, 17% for B4, 14% for B5 in 72 h. Vitality % values of HeLa cells treated with *C. umbrosum* are, respectively, 101% for U1, 70% for U2, 82% for U3, 52% for U4, 77% for U5 in 24 h; 99% for U1, 28% for U2, 19% for U3, 33% for U4, 21% for U5 in 48 h; and 63% for U1, 20% for U2, 21% for U3, 20% for U4, 23% for U5 in 72 h.

Several compounds derived from natural products, such as Genistein, Puerarin, Formononetin, Cyanidin 3-O-glucoside, Epigallocatechin gallate, Quercetin, Kaempferol, Hesperidin, Naringin, Apigenin 7-glucoside, Wogonin, Eugenol,

Anthocyanins, Curcumin, Emodin, Epifriedelanol, Mitomycin C, and Piperine, induce apoptosis in cervical cancers [6, 43]. Activation of apoptosis is used as an anticancer mechanism in cancer therapy research [6].

The determination of the AI is the method that helps to predict and diagnose patients by considering the tumor, its stage, the course of the disease, its consequences, the patient's strength of resistance, and the possibilities of helping the patient [44].

AI has been determined by DAPI respectively the fluorescence microscopies (**Figure 3**). DAPI staining was also conducted to show the apoptosis of HeLa cells. AI was calculated as follows (**Table 1**):

AI = (number of apoptotic cells/Total number counted) × 100%

Table 1.
AI calculation formula.

AI values of HeLa cells treated with *C. baytopiorum* and *C. umbrosum* for 24, 48, and 72 h are shown in **Figure 4**. AI values were determined as 2.3% for control group, 18.3% for B1, 16.4% for B2, 12.37% for B3, 25.12% for B4, 18.02% for B5 in the application of the *C. baytopiorum* extract; 8.66% for U1, 6.78% for U2, 14.49% for U3, 15.88% for U4, 18.91% for U5 in the application of the *C. umbrosum* extract in 24 h.

Bungu et al. [23] applied the prepared extracts from the leaves and bulbs of *Tulbaghia violacea* on HeLa, HT29, MCF-7, and WHCO3 cells. After 24 h, a significant number of apoptotic cells were detected. If after 48 h, a further increase in the number of apoptotic cells was observed. The results of our study are consistent with this and other similar studies.

In our experiments, in 24 h, it was determined significant cytotoxic effect according to the control ($p < 0.01$) in the experimental groups B1, B2, B4 and B5 applied the extract of *C. baytopiorum* and the experimental groups U3, U4 and U5 applied *C. umbrosum* extract.

AI values were determined as 2.84% for control group, 10.67% for B1, 35.91% for B2, 38.38% for B3, 22.04% for B4, 22.03% for B5 in the application of the C. *baytopiorum* extract; 8.9% for U1, 22.05% for U2, 30.19% for U3, 16.01% for U4, 23.58% for U5 in the application of the *C. umbrosum* extract in 48 h.

In our experiments, in 48 h, it was determined significant cytotoxic effect according to the control ($p < 0.01$) in the experimental groups B1, B2, B4 and B5 applied the extract of *C. baytopiorum* and the experimental groups U3, U4 and U5 applied *C. umbrosum* extract.

AI values were determined as 2.92% for control group, 7.89% for B1, 11.19% for B2, 11.76% for B3, 14.72% for B4, and 10.5% for B5 in the application of the *C. baytopiorum* extract; 2.82% for U1, 7.91% for U2, 15.22% for U3, 16.23% for U4, and 11% for U5 in the application of the *C. umbrosum* extract in 72 h.

In our experiments in B2, B3, B4, and B5 experimental groups applied *C. baytopiorum* extract and the U3, U4, and U5 experimental groups applied *C. umbrosum* extract was determined to have a significant cytotoxic effect according to control ($p < 0.01$) in 72 h.

The methanol extract of *C. baytopiorum* has been treated with the most effective concentration of 0.1 mg/ml in 48 h experiment group (after the evaluation of cytotoxicity and AI value).

The morphological changes belonging to the apoptosis such as blebbing of membrane and apoptotic body including cell organelles and chromatin parts [26, 45, 46] have been shown through phase-contrast, fluorescence, and light microscopies (**Figures 3** and **5–7**).

Most studies on apoptosis of plant extracts were confirmed by mechanisms such as caspase and *bcl-2* gene family [6]. A study showed that *Dendrobium chrysanthum*

ethanol extracts induct apoptosis by upregulated Bax, and p53 and downregulated Bcl-2 in HeLa cell lines at the density of 450 μg/ml for 24 h [47]. Another study reported that 0–100 μmol/L Wogonin induces apoptosis via elevating the Bax/Bcl-2 expression ratio, activating Caspase 3 and 9 in SiHa and CaSki cells [48].

To determine the apoptosis at the molecular level, the expression rates of the genes of the *bax*, *bak*, *bik*, *mcl-1*, *bfl-1*, *bcl2*, and *bcl-x*, which are members of the *bcl-2* gene family, have been searched through the RT-PCR method. In the 48 h experiment group of the HeLa cells that were treated with the 0.1 mg/ml concentration of the *C. baytopiorum* extract, an increase has seen in expressions of *mcl-1* and *bcl-x* genes compared to the control group, and some increase has been determined in the *bax*, *bak*, *bik,* and *bfl-1* genes expressions. On the other hand, no expression was observed in *bcl-2* gene (**Figure 8**).

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Conflict of interest

The authors declare no conflict of interest.

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