

## Research Article

# ***Bryonia aspera* Root Extracts Induce Programmed Cell Death in Selected Cell Lines of Glioblastoma, Ovarian, and Breast Cancer**

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**Background.** Medicinal plants have been remarkable sources of current chemotherapeutic agents. Ethnobotanical utilization of *Bryonia* species goes back to the old era, and contemporary but preliminary studies have evidenced the anticancer effects of this kind of plant. **Methods.** The MTT assay was used to investigate the cytotoxicity of a range of concentrations from different extracts of *Bryonia aspera* root in cancer and noncancer cells. The apoptosis was assessed using annexinV-FITC/PI flow cytometry assay. The expression of selected hallmark genes from different cell death modalities, including apoptosis, necroptosis, ferroptosis, and pyroptosis, was investigated using the qPCR method. The ROS production was also measured by the fluorescence technique. **Results.** Compared to the normal cells, all three extracts could induce significant cell death in lower doses in breast, ovarian, and glioblastoma cancer cells. Flow cytometry and gene expression studies revealed that different extracts of *Bryonia aspera* tend to induce different types of cell death in the selective cancer cell lines. ROS production was not impacted significantly by any of those three extracts in none of the cancer cells. **Conclusion.** The findings showed that all three extracts of *Bryonia aspera* root contain biologically active compounds that induce different types of programmed cell death in the investigated breast, ovarian, and glioblastoma cancer cells in concentrations significantly less than the doses affecting normal cells.

## 1. Introduction

Cancer is the second-leading global cause of human mortality [1]. With the limited rate of treatment efficacy and high recurrence rate due to late diagnosis and the presence of cancer stem cells, the finding of novel therapeutics is essential [2]. Apoptosis evasion is a hallmark of cancer and is not particular to the cause or kind of cancer. There are numerous mechanisms for cancer cells to avoid apoptosis: caspase function can be suppressed, or the apoptosis trigger can be silenced; hence, targeting it is useful for many types of cancer [3, 4]. In addition to overcoming apoptosis resistance, developing methods to induce non-apoptotic programmed cell death is essential and appealing as an alternative therapy for cancer [5]. Necroptosis, one of these types of cell death, is

a controlled caspase-independent form of cell death. In the absence of caspase 8 activity, RIPK1 binds and phosphorylates RIPK3, forming the ripoptosome complex. By enlisting and phosphorylating MLKL, the RIPK1/RIPK3 complex creates the necrosome. By mitochondrial instability, the RIPK1/RIPK3/MLKL pathway causes cell death [6]. Ferroptosis is a kind of programmed necrosis that occurs mostly through extra-mitochondrial lipid peroxidation triggered by iron-dependent ROS accumulation. Furthermore, PTGS2 (prostaglandin-endoperoxide synthase) and CHAC1 (glutathione-specific gamma-glutamylcyclotransferase 1) genes expression is a pharmacologic marker for ferroptosis. There are increasing numbers of studies being done on ferroptosis as a cancer treatment [7, 8]. Pyroptosis is a kind of cell membrane lysis-regulated cell death and inflammation

that calls for membrane-damaging gasdermin proteins. It is characterized by cell swelling and lysis as well as the release of numerous pro-inflammatory factors (including IL1 and IL-18). Pyroptosis can inhibit the occurrence and development of cancers. In regard to apoptosis resistance, stimulating pyroptosis in tumors offers significant therapeutic promise [9]. Several medicinal plants have anticancer activity, and some of them have been scientifically confirmed. The anticancer effect of medicinal plants or their bioactive components primarily targets cell cycle and cell death pathways [10, 11]. Chemotherapy undeniably plays a crucial role in the fight against cancer, and research has demonstrated that plant-based chemotherapy drugs can fulfill this function with anti-cancer therapeutic approaches while offering far fewer side effects. In this regard, the need to discover phytomedicines as a superior cancer treatment has been emphasized [12, 13]. Cucurbit plants have been widely utilized as traditional herbal treatments for a variety of diseases and have shown anti-inflammatory, anti-tumor, hepatoprotective, and immunomodulatory properties [14]. *Bryonia aspera*, from the cucurbitaceae family, contains a variety of cucurbitacins. Cucurbitacins are a class of triterpene-containing chemicals that are known to be poisonous and to inhibit cell growth [15]. It has been reported that active chemicals from various cucurbitaceae species could trigger different types of cell death [16–19]. According to ethnopharmacological information, the roots of *Bryonia aspera* Steven. ex Ledeb., locally known as “andaz” are used to treat cancer, liver problems, and digestive disorders in the Turkmen Sahra region in northern Iran [20].

The present study was carried out to investigate the potential of three different *Bryonia aspera* root extracts to induce various kinds of programmed cell death in U251-MG, A2780CP, MDA-MB231 cell lines, which represent glioblastoma, ovarian, and breast cancer.

## 2. Methods

**2.1. Plant Material.** The root part of plants was collected from the Turkmen Sahra, Golestan province, Iran, in autumn 2013 and after identification by a herbalist, a voucher specimen was deposited in the herbarium of Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

**2.2. Preparation of Extract.** The plant's root was dried in the shade, powdered, and consecutively extracted with solvents petroleum ether, dichloromethane and methanol by maceration and shaking constantly for 24 h. After that plant extract was filtered and remained solvent was evaporated using a rotary evaporator and stored at 4°C.

**2.3. Cell Culture.** Cell lines for glioblastoma (U251), ovarian cancer (A2780CP), breast cancer (MDA-MB231), and primary human dermal fibroblasts (HDFa) were provided from The SBMU School of Pharmacy. All cell lines were cultured in DMEM High Glucose (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 500 U/ml penicillin, and 500 µg/ml

streptomycin (PAN Biotech, Germany) in an incubator at a temperature of 37°C and 5% CO<sub>2</sub>.

**2.4. Cell Cytotoxicity.** The MTT assay was used to measure the cytotoxicity of various concentrations of different extracts of *Bryonia aspera* root in cancer and noncancer cells.  $0.1 \times 10^5$  cells were seeded into a 96-well plate and 24 h later cells were treated with different concentrations of extracts (0.1–1000 ng/µl) for 24 hours. After removing the media, MTT solution (5 mg/ml in PBS) was added to each well, and 4 h later DMSO was added to dissolve the violet formazan crystals that had formed within metabolically active cells. The absorption was measured at 570 nm (with 620 nm as the reference) using an ELISA reader. IC<sub>50</sub> values, as the concentration of extracts causing 50% inhibition of cell viability, were calculated using nonlinear regression curve in GraphPad prism 8.

**2.5. Cell Death.** Cell death was assessed using the AnnexinV-FITC/PI assay. Briefly, cancer cells were cultured into 6-well plates and treated with *B. aspera* root extracts for 24 h. Then, cells were washed with PBS and collected. 100 µl of the binding buffer including 5 µl Annexin V-FITC and 5 µl PI was added and incubated in the dark for 20 minutes.  $1 \times 10^4$  cells were counted for each sample. Apoptotic cells were quantified by a FACS Calibur flow cytometer (BD Biosciences, USA). Annexin-V positive and PI-negative cells were considered to be in the early apoptotic phase, whereas Annexin-V and PI-positive cells were considered to be in the late apoptotic or necrotic phase. The percentages of cells in each quadrant were analyzed using Flowing Software 2.5.1.

**2.6. RNA Isolation, Reverse Transcription, and Real-Time PCR.** Total RNA was isolated after 24 h treatment with *B. aspera* root extracts using YTzol pure RNA (YTA, Iran). The extracted RNA was checked for the quantity and quality using 260/280 nm absorption ratio and 1% agarose gel electrophoresis. cDNA synthesis was performed using the AddScript cDNA Synthesis Kit (AddBio, South Korea). For qPCR analysis of desired genes, the (SYBR, ROX) 2x Master mix kit (SYMBIO, Taiwan) was used. The qPCR reaction conditions included an activation step for 10 min 95°C followed by 40 cycles, including denaturation step for 15 s at 95°C, annealing step for 20 s at 60°C, and extension step for 25 s at 72°C. The specificity of qPCR products was verified by melting curve analysis, and the  $2^{-\Delta\Delta CT}$  method was used for gene expression quantification. The primers that were used are summarized in Table 1. The gene expression analysis results were obtained using REST software and GraphPad Prism 8.

**2.7. Reactive Oxygen Species Assay.** To assess ROS production in treated cells, the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used. DCFH-DA was dissolved in PBS at a 10 mM concentration. Cells were seeded in a 48-well plate. After the cells reached the required density, the culture medium was removed and

TABLE 1: Sequence of primers.

(PTGS2)	Forward primer	ATCACAGGCTTCCATTGACC
	Reverse primer	CAGACATTTCTTTCTCCTGT
(IL18)	Forward primer	TAGCTGAAGATGATGAAAACCTG
	Reverse primer	AATAGAGGCCGATTTCTTTGG
(CASP9)	Forward primer	CAAAGTTGTCGAAGCCAACC
	Reverse primer	TGGTCTTTCTGCTCGACATC
(GSDMD)	Forward primer	AACTTCTGACAGATGGGGT
	Reverse primer	CCAGTTCCTTGAGATGGTCTC
(CHAC1)	Forward primer	CTTGAAGATCATGAGGGCTG
	Reverse primer	AGGAGCATCTTGGGGATAGA
(CASP3)	Forward primer	TAGCGGATGGGTGCTATTG
	Reverse primer	CTTCCATGTATGATCTTTGGTTC
(RIPK1)	Forward primer	CCTTGCCACCAACAGATGAA
	Reverse primer	GAACTCGTCCCACCAATCTC
(RIPK3)	Forward primer	TGCGTCAAGTTATGGCCC
	Reverse primer	CATCGTAGCCCCACTTCCTA
(MLKL)	Forward primer	GCCCTCTGTGGATGAAATCTT
	Reverse primer	GATAACCCAATGCCGAAGGA
(B2M)	Forward primer	TGTCTTTCAGCAAGGACTGGT
	Reverse primer	TGCTTACATGTCTCGATCCCA

washed with PBS. Afterward, 200  $\mu\text{l}$  of DCFH-DA reagent and 2  $\mu\text{l}$  of Hoechst 33342 dye were added to each well. The substrate solution was removed after 1 h of incubation at 37°C, and cells were treated with *B. aspera* root extracts. Absorption was measured with the Cytation 5 Cell Imaging Multimode Reader device at wavelengths of 450 nm and 530 nm for ROS and 350 nm and 460 nm for Hoechst at different time periods (30 min –1 h –3 h –6 h and 24 h). According to the positive control of 1% H<sub>2</sub>O<sub>2</sub> and the negative control of N-acetylcysteine (3 mM), the induction and inhibition of oxidative stress in this test were validated. The ROS levels were expressed as RFU (Relative Fluorescence Unit).

**2.8. Statistical Analysis.** Two-way analysis of variance (ANOVA) and Tukey's post hoc were applied for data analysis. *P* values <0.05 were considered statistically significant.

### 3. Results

**3.1. Cytotoxicity Assay.** As shown in Figure 1, the cytotoxic activity of methanol, dichloromethane and petroleum ether extracts from *Bryonia aspera* root in different concentrations ranging from 0.1 to 1000 ng/ $\mu\text{l}$  was evaluated using an MTT assay. The IC<sub>50</sub> values of methanol extract in U251, A2780CP, MDA-MB231, and HDF, as normal cells, after 24 h treatment were approximately 30.18, 113.2, and 104.4 ng/ $\mu\text{l}$ , and 229.8 ng/ $\mu\text{l}$  respectively. The IC<sub>50</sub> values of dichloromethane extract in U251, A2780CP, MDA-MB231, and HDF cell lines were approximately 111, 100.4, 94.46, and 165 ng/ $\mu\text{l}$ , respectively, and the IC<sub>50</sub> values of petroleum ether extract in U251, A2780CP, MDA-MB231, and HDF cell lines were approximately 104.2, 149.2, 137.2, and 216.4 ng/ $\mu\text{l}$ , respectively. These findings indicated that these

extracts had a lower cytotoxic effect on normal cells than on cancer cell lines. The concentration of 100 ng/ $\mu\text{l}$  was used for the rest of the investigation. Except for the methanol extract in the U251 cell line, which was chosen at a concentration of 30 ng/ $\mu\text{l}$ .

**3.2. Cell Death Assay.** Annexin V/PI staining was used to measure the cell death modality. After 24 h treatment with methanol, dichloromethane, and petroleum ether extracts of *B. aspera*, results showed that all three extracts could induce apoptosis in U251, A2780 CP, and MDA-MB231 cell lines (Table 2). As shown in Figure 2, methanol, dichloromethane, and petroleum ether extracts of *B. aspera* root induced apoptosis  $19.82 \pm 1.78$ ,  $6.75 \pm 1.08$ ,  $5.7 \pm 0.9$  times more than control group, respectively, in U251 cell line ( $p < 0.0001$ ). As indicated in Figure 3 Methanol, dichloromethane and petroleum ether extracts increased apoptosis  $5.51 \pm 0.81$ ,  $1.90 \pm 0.28$ , and  $2.95 \pm 0.43$  times more than the control group in A2780CP cell line ( $p < 0.0001$ ). As shown in Figure 4 methanol, dichloromethane, and petroleum ether extracts increased apoptosis  $4.23 \pm 0.63$ ,  $12.86 \pm 1.92$  and  $6.44 \pm 0.96$  times more than control group in MDA-MB231 cell line ( $p < 0.0001$ ).

**3.3. Gene Expression Assay.** As it shown in Figure 5 the expression level of CASP3, CASP9, RIP3K, MLKL, and CHA1C was increased whereas there was a reduction in expression of IL18 following 24 h of treatment with methanol (MET) extract of the *B. aspera* in U251 cell line ( $p < 0.0001$ ). After 24 h treatment with dichloromethane (DCM) extract, expression of CASP3, CASP 9, RIP3K, PTGS, and CHA1C were increased but the expression of GSDMD was reduced ( $p < 0.0001$ ) Following 24 h of treatment with petroleum ether (PE) extract, there was an increase in expression of CASP3, CASP9, RIP3K, PTGS, and

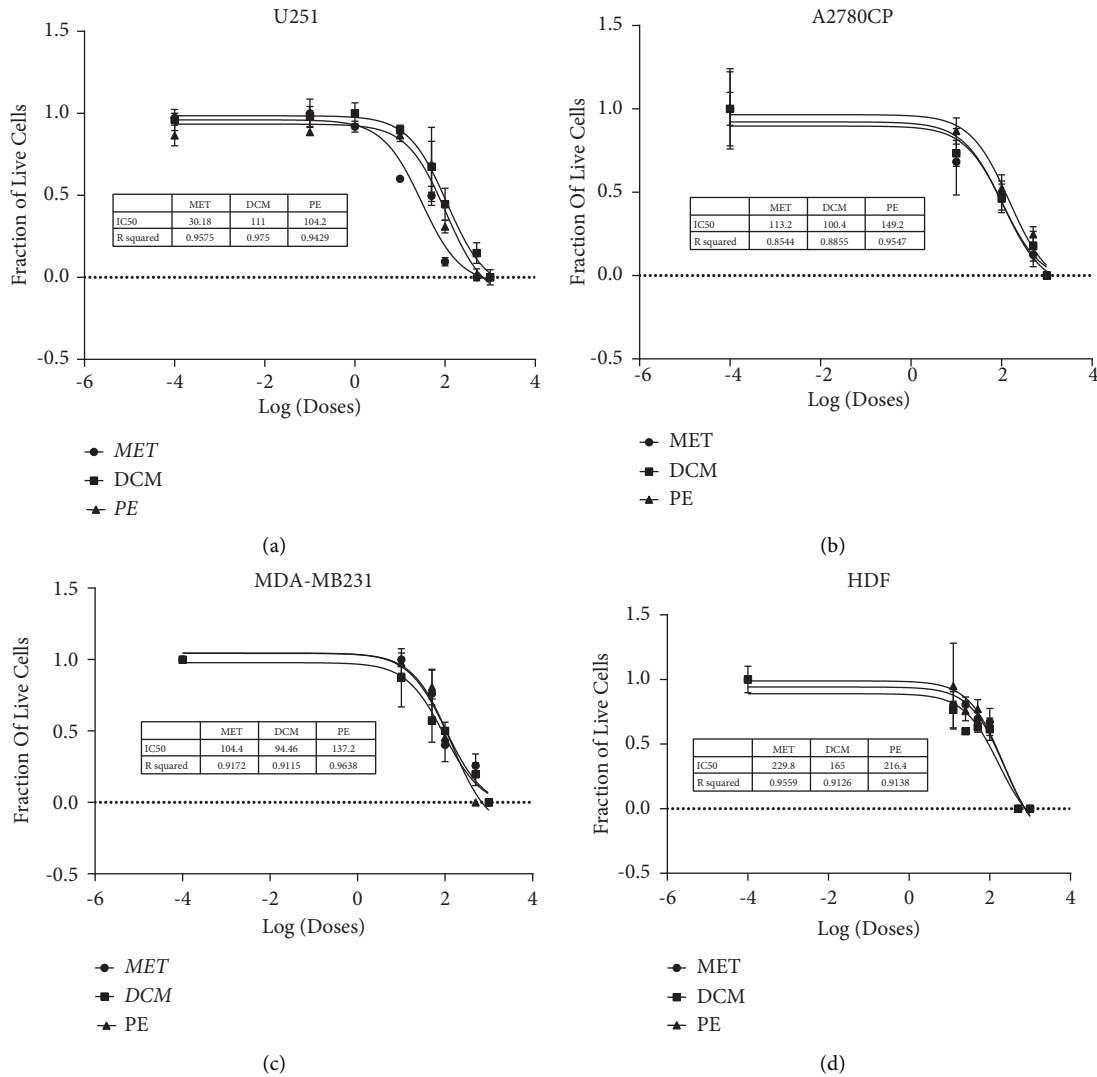


FIGURE 1: Cytotoxicity of three different *B. aspera* extracts on U251 (a), A2780CP (b), MDA-MB231 (c) and HDF (d) cell lines, evaluated by MTT assay. IC<sub>50</sub> is expressed as mean  $\pm$  SD ( $n = 3$ ).

TABLE 2: Apoptosis induction by different *B. aspera* root extracts in U251, A2780 CP, and MDA-MB231 cell lines compared to the control group.

Cell line	Extract	Fold change of apoptosis (mean $\pm$ SD)
U251	Methanol	19.82 $\pm$ 1.78
	Dichloromethane	6.75 $\pm$ 1.08
	Petroleum ether	5.7 $\pm$ 0.9
A2780 CP	Methanol	5.51 $\pm$ 0.81
	Dichloromethane	1.90 $\pm$ 0.28
	Petroleum ether	2.95 $\pm$ 0.43
MDA-MB231	Methanol	4.23 $\pm$ 0.63
	Dichloromethane	12.86 $\pm$ 1.92
	Petroleum ether	6.44 $\pm$ 0.96

CHA1C but a reduction in expression of IL18 ( $p < 0.0001$ ). Results indicated an increase in expression of CASP 3, CASP 9, RIP3K, MLKL, and downregulation in RIP1K, PTGS and IL18 expression ( $p < 0.0001$ ) after 24 h treatment with MET

extract in A2780CP cell line (Figure 6). After 24 h treatment with DCM and PE extracts, expression of CASP3, MLKL and RIP3K were increased ( $p < 0.0001$ ). DCM extract reduced expression level of PTGS gene ( $p < 0.0001$ ). Findings in Figure 7 showed increased expression of CASP 3, RIP1K, RIP3K, MLKL, GSDMD and IL18 after 24 h treatment with Met extract in MDA-MB231 ( $p < 0.0001$ ). There was upregulation of CASP3 and CASP 9, but downregulation of RIP1K and RIP3K after treatment with DCM extract ( $p < 0.0001$ ). Expression of CASP 3, CASP 9 and CHA1C were increased after treatment with PE extract ( $p < 0.0001$ ).

**3.4. ROS Production Assay.** Oxidative stress was measured after treatment with MET, DCM and PE extracts of *B. aspera* in U251, A2780CP and MDA-MB231 cancer cell lines. The findings in Figure 8 showed no significant induction in ROS production in cells treated with extracts compared to the untreated cells.

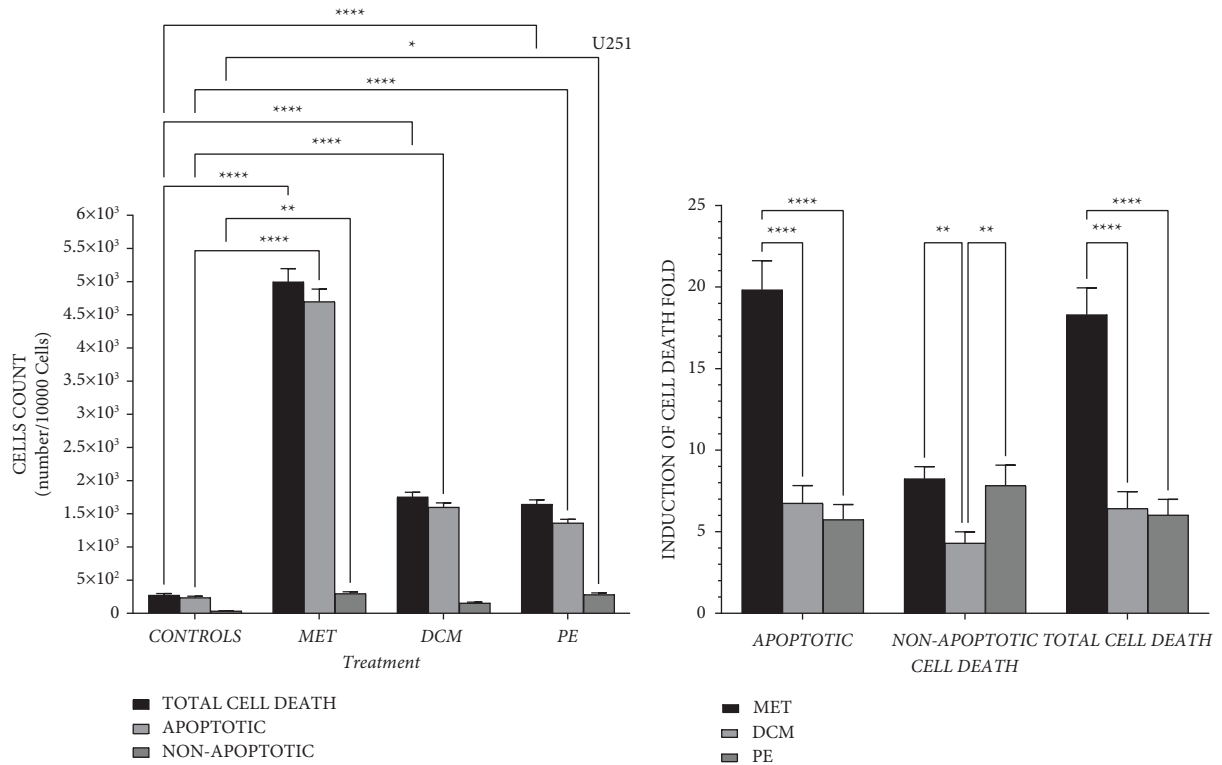


FIGURE 2: Comparison of the proportion of apoptotic cells in the treated cells and the untreated cells and coefficient of cell death after treatment with a 30 ng/ml concentration of methanol and 100 ng/μl concentration of dichloromethane and petroleum ether extracts in U251 cell line.

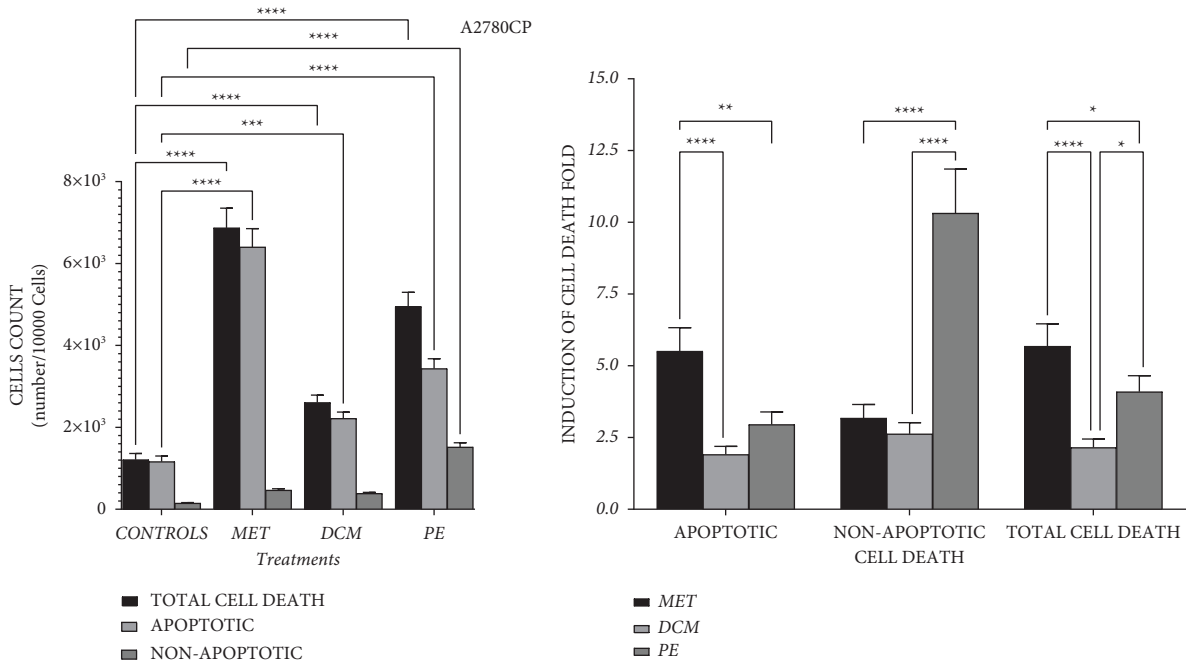


FIGURE 3: Comparison of the proportion of apoptotic cells in the treated cells and the untreated cells and coefficient of cell death after treatment with 100 ng/μl concentration of methanol dichloromethane and petroleum ether extracts in A2780cp cell line.

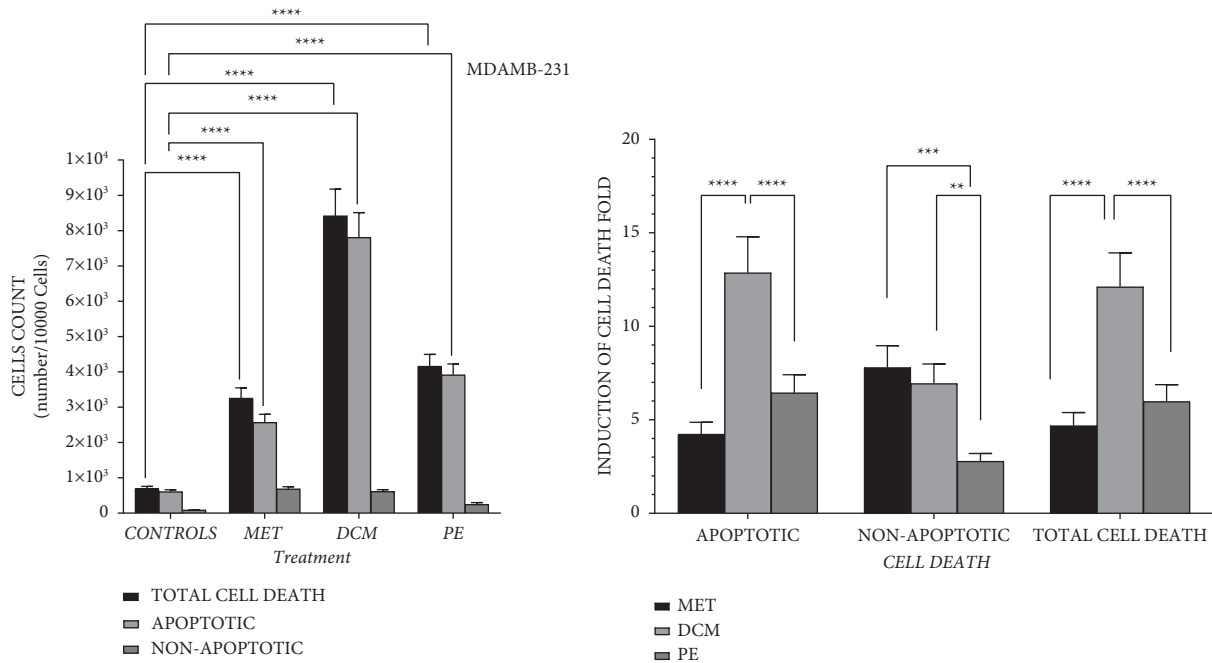


FIGURE 4: Comparison of the proportion of apoptotic cells in the treated cells and the untreated cells and coefficient of cell death after treatment with 100 ng/μl concentration of methanol dichloromethane and petroleum ether extracts in MDA-MB231 cell line.

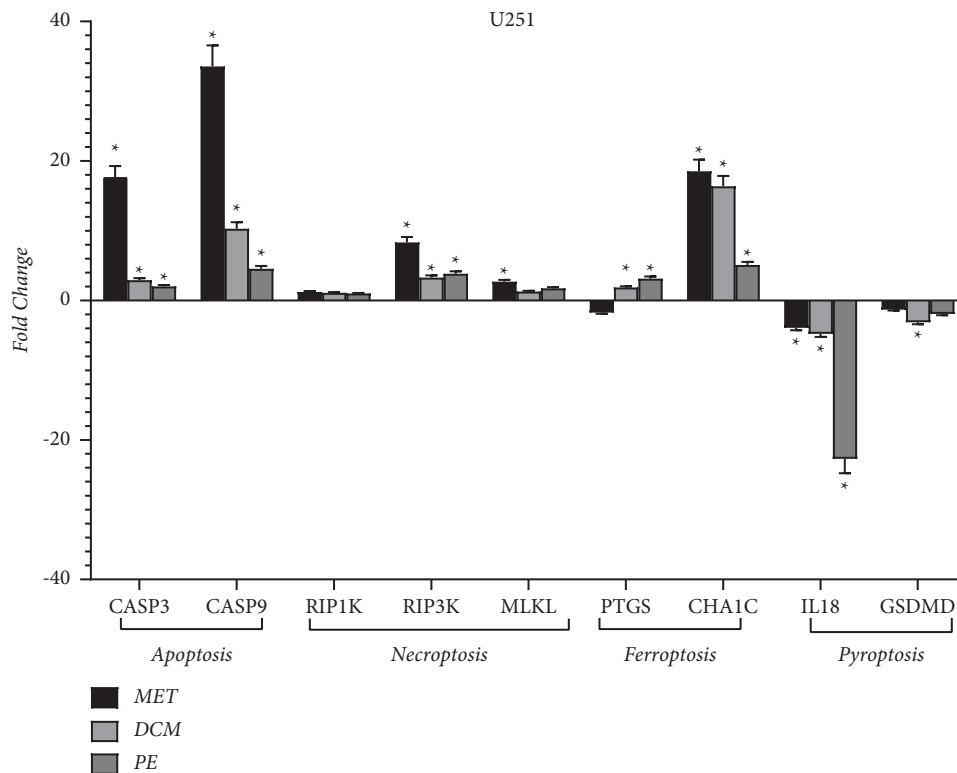


FIGURE 5: The expression of apoptosis, necroptosis, ferroptosis and pyroptosis genes in U251 cell line after 24 h treatment with 30 ng/μl concentration of methanol and 100 ng/μl concentration of dichloromethane and petroleum ether extracts.

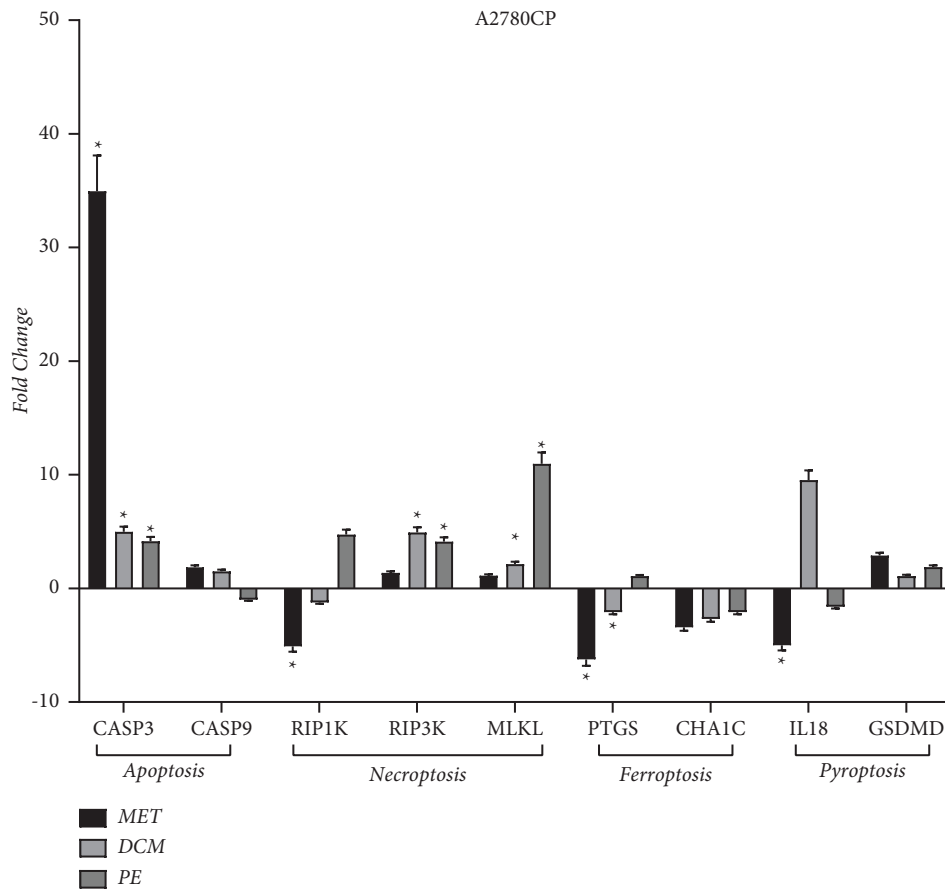


FIGURE 6: The expression of apoptosis, necroptosis, ferroptosis and pyroptosis genes in A2780cp cell line after 24 h treatment with 100 ng/ $\mu$ l concentration of methanol, dichloromethane and petroleum ether extracts.

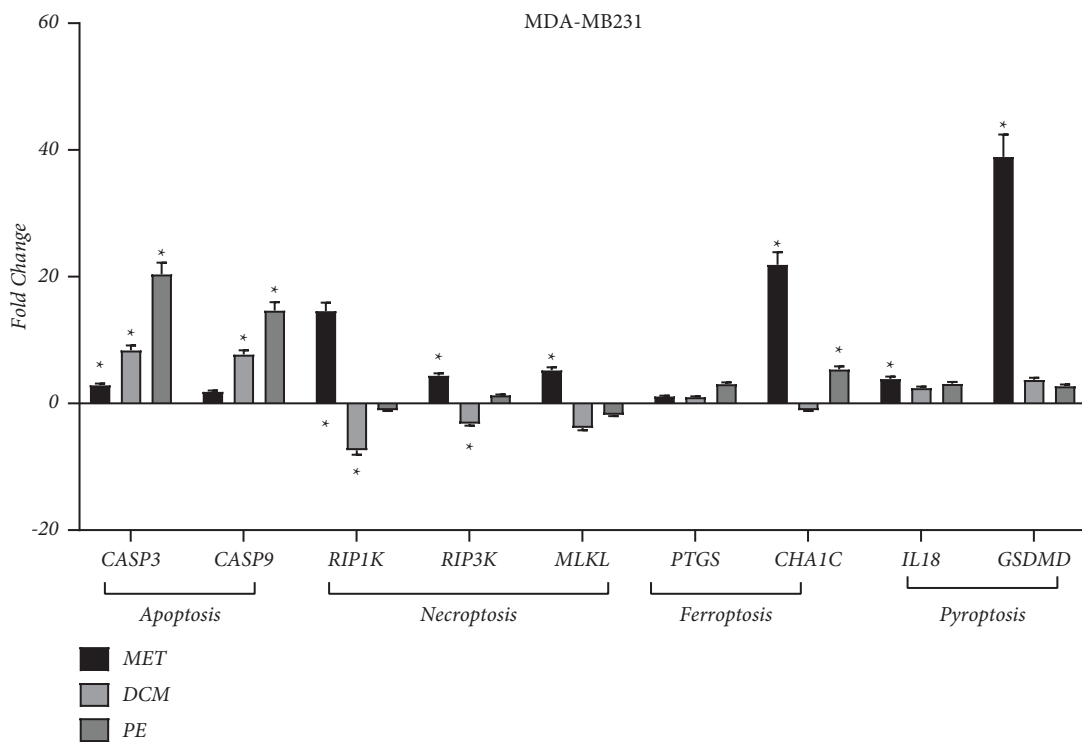


FIGURE 7: The expression of apoptosis, necroptosis, ferroptosis and pyroptosis genes in MDA-MB231 cell line after 24 h treatment with 100 ng/ $\mu$ l concentration of methanol, dichloromethane and petroleum ether extracts.

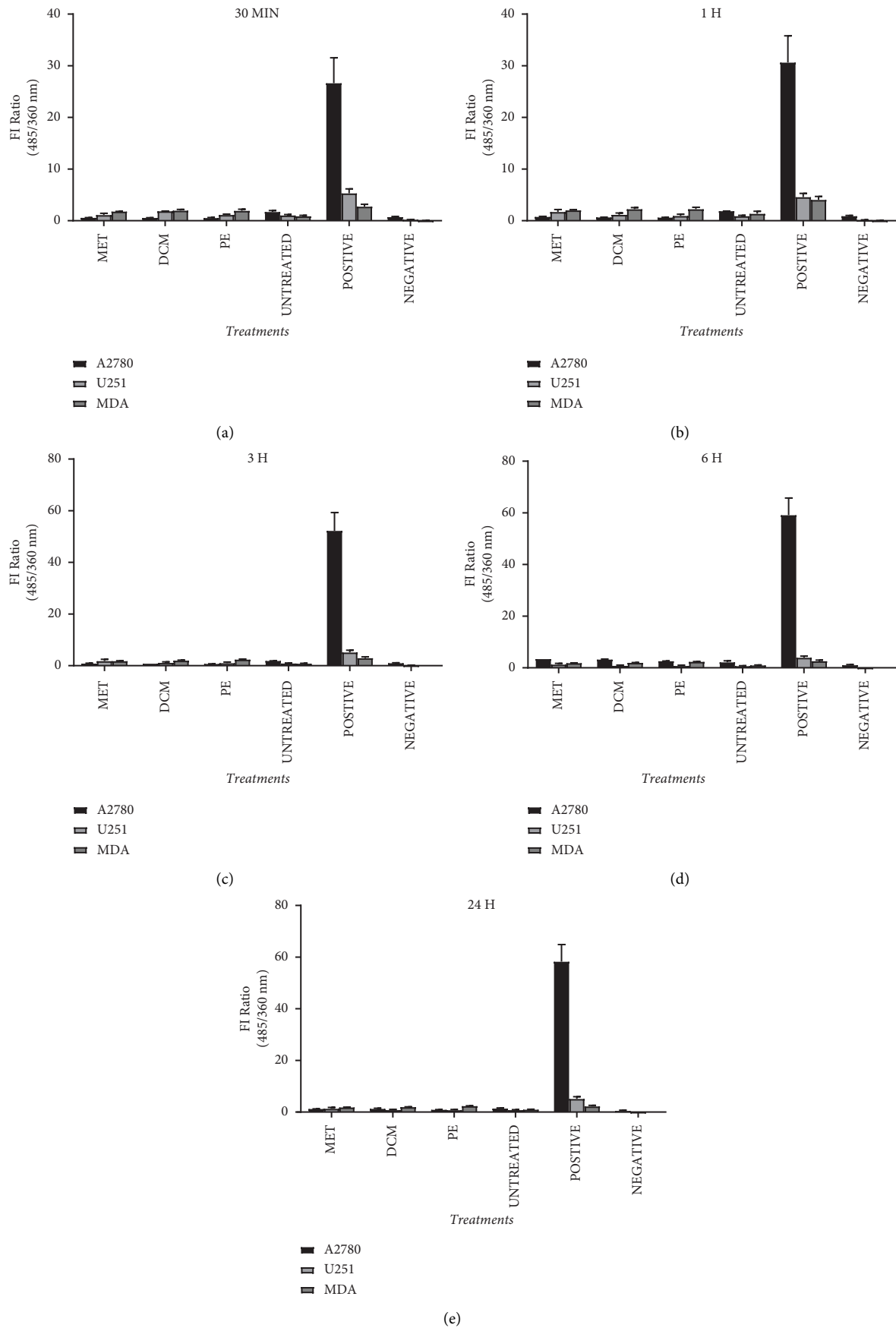


FIGURE 8: The effects of three extracts on ROS generation at different time periods (a, b, c, d, e) in cancer cell lines.



#### 4. Discussion

Most drugs used in the treatment of various diseases are derived from plant compounds, which are later produced on an industrial basis [21]. Preclinical research has focused on medicinal plant-based compounds that have the potential to treat cancer. [12] Apoptosis induction is one of the most crucial factors of cytotoxic chemotherapeutic agents. Natural compounds induce apoptotic pathways that are inhibited in cancer cells via various mechanisms [13, 22]. *Bryonia aspera*, belongs to the genus *Bryonia*, contains different type of cucurbitacins. Cucurbitacins are a class of compounds with a triterpene structure, known to possess toxicity and to suppress proliferation in various ways in cancer cell lines (B, D, E, I) [15]. For instance, Cucurbitacin B, a purified compound, has been reported to have the ability to cause cell death in both in vitro and in vivo models [18]. In this study, the findings showed that three extracts (methanol, dichloromethane, and petroleum ether) of *Bryonia aspera* root had cytotoxic activity against breast, ovarian, and glioblastoma cancer cells in concentrations significantly less than the toxic dose on normal cells which is in line with Sahranavard's research [23]. Furthermore, the anti-proliferative activity of cucurbitacins B, D, E, and I have been proven in colon (HCT-116), breast (MCF-7), lung (NCI-H460), and brain (SF-268) cancer cell lines, with cucurbitacin B demonstrating more than 80% growth inhibition. In lung cancer cells, cucurbitacins A, B, E, I, and Q had an antiproliferative effect. Cucurbitacin I inhibited the growth of breast and prostate cancer cell lines (MDA-MB-231, MDA-MB-468, and Panc-1), both in vitro and in xenograft models on nude models [24].

Necroptosis (a controlled and caspase-independent cell death process) can overcome apoptosis resistance and may promote and increase antitumor immunity in cancer therapy [25]. The results showed that Met extract of *B. aspera* could increase non-apoptotic death, which was in favor of necroptosis. This is in addition to the significant induction of apoptosis in the flow cytogram and the rise in the expression of the caspase 3 and 9 genes. The expression of the major necroptosis genes increased in U251 and A2780cp cancer cell lines after treatment with dichloromethane extract of *B. aspera*. It has been reported that active compounds from different cucurbitaceae species could trigger necroptosis in neuroblastoma cells [16].

According to flow cytometry analysis of cell death in three cancer lines treated with dichloromethane extract, the MDA-MB231 cell line had the highest rate of apoptotic death, compared to other programmed cell deaths, which was followed by an increase in the expression of both caspase 9 and 3 genes. In other cucurbitaceae, such an effect in inducing apoptotic death has been reported in the MDA-MB231 cell line [26].

Pyroptosis (regulated cell death) is characterized by cell membrane lysis and inflammation. There is significant therapeutic potential for the treatment of cancer in the stimulation of pyroptosis in tumours, particularly in apoptosis resistance [9]. The effect of DCM extract on promoting non-apoptotic death through analyzing the

expression of selected genes revealed that this extract increased the expression of pyroptosis genes in the MDA-MB231 cell line. It has been reported in another study that cucurbitacin B (CuB) inhibited tumour growth in non-small cell lung cancer (NSCLC) and NSCLC mice by inducing pyroptosis [17]. In our study, there was no noticeable difference between the sensitivity of three cancer cell lines to the petroleum ether extract. The activation of apoptotic genes was more in the MDA-MB231 cell line than in the other two lines, but the important feature of this extract was the stronger induction of non-apoptotic death rather than apoptotic death, which is more oriented towards increasing the expression of necroptosis and ferroptosis genes [27].

Ferroptosis is defined as “programmed necrosis” which is primarily triggered by extra-mitochondrial lipid peroxidation driven by iron-dependent ROS accumulation. The number of studies focusing on ferroptosis as a cancer treatment is growing [7]. Active ingredients of cucurbitacins such as CuB has been reported to have therapeutic potential as a ferroptosis-inducing agent for nasopharyngeal carcinoma [19]. The analysis of the generation of oxidative stress revealed that none of the three extracts significantly increased generation of oxidative stress in any of the cell lines between 30 minutes and 24 hours after treatment. The research done on several cucurbitaceae species was more in favor of the antioxidant activity of these compounds, which has been reported in previous studies [28, 29]. However, in other studies it has been reported that cucurbitacins such as cucurbitacin I induce apoptosis through increasing intracellular reactive oxygen species in SKVO3 (ovarian cancer) cells [30].

Our study is the first to reveal that different *B. aspera* root extracts possess greater lethal potential in breast (MDA-MB231), ovarian (A2780 CP), and glioblastoma (U251) cancer cell lines compared to normal human cell line (HDFa). Moreover, different *B. aspera* root extracts can induce programmed cell deaths in the investigated breast, ovarian, and glioblastoma cancer lines. However, further studies are required to evaluate *B. aspera* root extracts as potential chemotherapeutic agent in cancer treatment.

#### 5. Conclusion

The findings showed that all three extracts of *Bryonia aspera* root contain biologically active compounds that induce different types of programmed cell death in the investigated breast, ovarian, and glioblastoma cancer cell lines in concentrations significantly lower than those affecting normal cells. Furthermore, the three extracts did not increase the production of oxidative stress, so both apoptotic and non-apoptotic effect occur through mechanisms independent of the oxidative stress-dependent pathway.

#### Data Availability

The data that support the findings of this study are available on request from the corresponding author.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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