

# Antiproliferative and Apoptotic Effects of Red Beetroot and Betanin on Human Colorectal Cancer Cell Lines

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## Research

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# Abstract

**Background:** Colorectal cancer (CRC) is the third most common type of cancer worldwide. Fruit and vegetables have some active compounds such as flavonoids and polyphenols that protect against malignancies through their antioxidative, anti-inflammatory, anti-proliferative, neuro, and hepatoprotective properties. Red beetroot (*Beta vulgaris*) contains red (betacyanins) and yellow (betaxanthins) pigments known as betalains. Betanin makes up 75-95% of the total betacyanins, possessed a wide range of favorable biological effects such as chemopreventive, anticarcinogenic, anti-tumorogenic, antiangiogenic, and proapoptotic effects.

**Methods:** Red beetroot hydro-alcoholic extract and betanin were used to treat Caco-2 and HT-29 colorectal cancer cells, as well as KDR/293 normal epithelial cells. The half-maximal inhibitory concentration (IC<sub>50</sub>) was determined by prescreening MTT tests in the range of 20 to 140 µg/ml at 24 and 48 h. The cytotoxicity and apoptosis-inducing evaluations were performed via MTT assay, DAPI staining, and FACS-flow cytometry tests using determined times and doses. Moreover, the expression level of six important genes involving in the apoptosis pathway (Bcl-2, BAD, Caspase-3, Caspase-8, Caspase-9, and Fas-R) were determined using the real-time polymerase chain reaction (RT-PCR) method.

**Results:** The IC<sub>50</sub> doses for HT-29 and Caco-2 cell lines were determined to be about 92 µg/mL, 107 µg/mL for beetroot hydro-alcoholic extract, and 64 µg/mL, 90 µg/mL for betanin at 48 h, respectively. Our findings showed that beetroot extract and betanin significantly inhibit the growth of HT-29 and Caco-2 cell lines, time and dose-dependently, without considerable adverse effects on KDR/293 normal cells. Moreover, DAPI staining and flow cytometry results revealed significant apoptosis symptoms in treated cancerous cell lines. The expression level of pro-apoptotic genes involved in intrinsic and extrinsic apoptosis pathways (BAD, Caspase-3, Caspase-8, Caspase-9, and Fas-R) in treated HT-29 and Caco-2 cells was higher than untreated and normal cells, whereas the anti-apoptotic gene (Bcl-2) was downregulated.

**Conclusion:** Beetroot hydro-alcoholic extract and betanin significantly inhibited cell proliferation and induced cell apoptosis (intrinsic and extrinsic pathways) via modification of effective genes in both colorectal cancer cell lines with no significant cytotoxic effects on KDR/293 normal cells. The mechanism of the anticancer effects of red beetroot extract and betanin needs to be further studied.

## Background

Colorectal cancer (CRC) is the third most common type of cancer in all age groups accounting for approximately 1.2 million new cases and in the last years its incidence and mortality rate increased among young adults (1, 2). Although age and male sex are two important risk factors of CRC but based on different epidemiological studies some other inherent and environmental factors such as inflammatory bowel disease, smoking, excessive alcohol consumption, high consumption of red and processed meat, obesity and diabetes have been identified (2, 3). Diet and lifestyle as two key

environmental factors are accepted as major contributors to CRC development while numerous investigations suggested that 30–35% of cancer cases are avoidable through the following of healthy diet, regular physical activity and keeping a healthy body weight (4–7). Several studies have demonstrated a reverse relationship between fruits and vegetable's consumption of with risk of chronic diseases and malignancies (8–11). Based on confirmed results, fruits and vegetables as rich sources of bioactive compounds with high antioxidant capacity attracted much attention in human nutrition and medicine. In this way, recognized dietary antioxidants such as ascorbic acid, R-tocopherol, carotenoids, anthocyanins (flavonoid glycosides), and recently betalains (12–14) have been targeted by different investigators due to their high potential capacity to scavenge free radicals that lead to different cellular damage. (13, 15, 16). Red–violet betacyanins (e.g., betanin and isobetanin) and yellow betaxanthins (e.g., vulgaxanthin I and II) are two water-soluble nitrogenous pigments that exist in most members of plant order Caryophyllales (except the Caryophyllaceae and Molluginaceae) and in some higher fungi (14, 17). Red beetroot (*Beta vulgaris* L.) has eminent antioxidant capacity due to the presence of betalains composed of red pigments (betacyanins) and yellow pigments (betaxanthins) (18). Betalains as the main constituent of red beetroot mostly consisted of betanin, isobetanin, and vulgaxanthin I & II and are used as a natural colorant in numerous food industries such as dairy products, beverages, candies, and cattle products. (19–22). Betanin is the water-soluble nitrogenous compound and comprises 75-95% of red beetroot pigments (300-600 mg/kg) (Fig. 1) (16–18, 23). This active phytochemical possesses various favorable biological effects including antioxidant, anti-inflammatory, hepatoprotective, and antitumor activities through its aromatic amino compound moieties that are excellent electron donors that stabilize free radicals (13, 14). It has been demonstrated that beetroot and its main constituent, betanin, can be effective as a strong chemopreventive agent that induces apoptosis and decreases cell proliferation, angiogenesis, inflammation in skin, liver, lung, and esophageal cancer in experimental animals and cancer cell lines (24–28). Also, beetroot extract showed effective cancer chemopreventive activity and inhibited the carcinogenic effects of 7, 12-imethylbenz[a]anthracene, and diethylnitrosamine that induced skin cancer and hepatocarcinogenesis in mice (27, 29). Besides, it was shown that treatment of human chronic myeloid leukemia cell line-K562 with 40mM betanin decreases cell proliferation (50%) and induces intrinsic apoptosis pathway by activation of caspase-3 as an executioner caspase in apoptotic cascades (30, 31). Based on the fact that red beetroot main constituent, betanin, has potent antioxidant, antiproliferative, antitumor activities and also inhibits initiation and promotion steps of chemical carcinogenesis, it is suggested that beetroot is a useful cancer preventive vegetable that can be effective in preventive and therapeutic medicine (25, 27). However, there are few studies that have examined the anti-cancer effects of beetroot extract and betanin in colorectal cancer. As well, there is limited information about molecular mechanisms of anticancer activity of beetroot and betanin. Thus, the aim of the present study was to further explore and compare the mechanism of anticancer activity of beetroot hydro-alcoholic extract and its major component, betanin, on human colorectal cancer cell lines (Caco-2, HT-29) in comparison with normal epithelial cells (KDR/293). Furthermore, to identify the exact mechanism of its apoptosis-inducing effects, we assessed the expression level of 6 key genes (Bcl-2, BAD, Fas-R, Caspase-3, Caspase-8 and Caspase-9) that play important roles in intrinsic and extrinsic apoptosis pathways.

## Methods

### Plant Material

The mature beetroots were obtained from a local market in Tabriz city, Iran. Leaves were removed and soil cleaned from the roots and washed several times with sterile water, then clean roots were chopped and used for hydro-alcoholic extraction.

### Beetroot hydro-alcoholic extraction

One kilogram of fresh chopped beetroots was homogenized via blender. Then, the obtained liquid, extracted three times with 70% ethanol (100 mg/L). The extract was centrifuged at 10,000 g for 30 min and the supernatant was evaporated at 40°C under vacuum until drying. The residual aqueous was dissolved in 1000 mL of 70% methanol (300 mL water/700 mL methanol), then the methanol-sample mixture was refrigerated at -20°C for 24 h, and the supernatant was carefully collected from the precipitate. The methanol was removed from the supernatant by evaporation at 40°C under vacuum, then the aqueous fraction was lyophilized (Christ, Alpha 1-2, Germany) and used as dry beetroot extract. The total amount of obtained final dry extract from 1 kg biomass was 14.5 g/1kg.

### Cell lines and culture medium

Two human colorectal cancer cell lines (Caco-2, ATCC, HTB-37 and HT-29, ATCC, HTB-38) and a human epithelial normal cell line with the same embryonic origin (KDR/293) were cultured in 25 cm<sup>2</sup> culture T-flasks in Roswell Park Memorial Institute medium (RPMI 1640, Sigma, Poole, United Kingdom) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) respectively under standard conditions at 37 °C and 5% CO<sub>2</sub>. All media were supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA), 1% of mixture penicillin (100 IU/ml) (Sigma, St. Louis, MO, USA), and streptomycin (100 µg/ml) (Sigma, St. Louis, MO, USA).

### MTT assay

MTT test was performed on untreated and treated cell lines with beetroot extract, betanin (Sigma-Aldrich Chemie GmbH) and 5-Fluorouracil (5-FU) (7µl/well) as described in our previous studies (32, 33). The half maximal inhibitory concentration (IC<sub>50</sub>) were determined in HT-29 and Caco-2 cells by prescreening MTT tests (in the range of 20 to 140 µg/ml) at 24 and 48 h (Fig. 2). The absorbance was measured using ELISA plate reader (Biotek, ELx 800, USA) at 570 nm. The growth inhibitory effects of supernatant were calculated according to the following formula: the Growth Inhibition Ratio (IR %) = [(the absorbance of blank control group – the absorbance of experimental group)/the absorbance of blank control group] × 100 %.

### Morphological analysis

DAPI staining method was used for finding morphological changes regarding all treated and untreated groups with beetroot extract, betanin, and 5-Fluorouracil (5-FU) as exactly described in our previous

studies (32, 33).

## Flow cytometry assessment

Colorectal cancer cell lines (HT-29 and Caco-2) and normal KDR/293 cells were seeded into six-well culture plates ( $1.2 \times 10^5$  cells/well) and treated with beetroot extract, betanin, and 5-Fluorouracil (5-FU). After treatment time point, the treated/untreated cells were detached by Trypsin-EDTA (Sigma-Aldrich, St Louis, MO), and the liquid phase including beetroot extract and betanin was discarded after centrifugation at 900 rpm for 10 min at 28°C. In accordance with kit instructions (eBioscience, San Diego, CA), the cell pellets were washed by PBS and 1X Binding Buffer (1 mL 10X Binding Buffer + 9 mL dH<sub>2</sub>O) then centrifuged and their supernatants were thrown away. Afterward, 5 µl of FITC-conjugated Annexin V were added to 100 µl of the cell suspension and incubated for 15 min at room temperature under dark conditions. Finally, 5 µl of propidium iodide staining solution was added to the cells and flow cytometry assessment was performed on all untreated/treated cells as exactly described in our previous studies (32, 33).

## RNA isolation, complementary DNA synthesis, and real-time PCR analysis

All treated/untreated cells were washed 3 times with sterile PBS (pH 7.2). Total RNA was isolated from cells by direct lysis using RNX-plus solution according to manufacturer's instruments. The obtained total RNA pellet was dissolved in 50µL DEPC-treated water, and then quantity and quality of total RNA was assessed by UV spectrophotometry and agarose gel electrophoresis, respectively. One microgram of isolated RNA was used for synthesis of complementary DNA (cDNA) using Prime Script RT Reagent kit according to manufacturer's recommendations for cDNA synthesis. To amplify intended genes, particular primers, listed in the Table 1, were designed for each gene. All amplification reactions were performed in triplicate for each sample, and every experiment mixture (20 µL), containing 10 µL SYBR Green PCR master mix, 1 µL cDNA (1 µg/µL), 1 µL primer (forward and reverse), and 0.8 µL 6-carboxy-X-rhodamine (ROX as reference dye), was subjected to ABI-step 1 plus (Applied Biosystems, Foster City, CA, USA) instrument (34, 35). Thermal cycling condition was as follows: 1 cycle at 95°C for 5minutes followed by 40 cycles at 95°C for 20 seconds, 60°C for 35 seconds, and 72°C for 10 seconds.

Table 1  
Primers sequences for RT-PCR amplification.

Gene name and symbol	Sequence (5' → 3')	Amplicon size (bp)	TM	
			F	R
Bcl-2	F:5'-GGTGGGGTCATGTGTGTGG-3' R:5'-CGGTTCAGGTAAGTCAAGTCAATCC-3'	89	60.6	60.1
BAD	F:5'-TGGACTCCTTTAAGAAGGGAC-3' R:5'-CAAGTTCCGATCCCACCAG-3'	113	56.6	57.8
Fas R	F: 5'-AGCGCTGAAGAGCCAACATA-3' R: 5'-TGGGTAAGTATGATGCAACT-3'	126	59.7	58.7
Caspase- 3	F:5'-TGCCTGTAAGTGGAGAGTAGATGG-3' R:5'-CTTCACTTTCTTACTTGGCGATGG-3'	172	59.8	60.1
Caspase- 8	F: 5-ACATGGACTGCTTCATCTGC-3' R:5'-AAGGGCACTTCAAACCAGTG-3'	123	58.2	58.6
Caspase- 9	F:5'-TGCTGCGTGGTGGTCATTCTC-3' R:5'-CCGACACAGGGCATCCATCTG-3'	94	63.2	63.1
GAPDH	F:5'-AAGCTCATTTCTGGTATGACAACG-3' R:5'-TCTTCCTCTTGTGCTCTTGCTGG-3'	126	61.6	62.6

Interpretation of the results were performed using Pfaffle method and the threshold cycle (Ct) values were normalized to the expression rate of GAPDH as a housekeeping gene (36). All of the reactions were performed in triplicate and negative controls were included in each experiment.

## Statistical Analysis

The statistical analysis was performed using the statistical package for the social sciences (SPSS Inc. Chicago, IL, USA version 16.0). The Kolmogorov-Smirnov test was applied to evaluate the normal distribution of data. One-way ANOVA and Tukey's post hoc test were used for analyzing of differences between all treatments and multiple mean comparisons, respectively. Statistical significance was considered as a value of  $P \leq 0.05$  and quantitative data were reported as means  $\pm$  SD.

## Results

# Cell viability assay in cancerous and normal cell lines

After treatment with different doses (20 to 140 µg/ml) of beetroot extract and betanin in two time-points (24 and 48 h), the IC<sub>50</sub>s were determined as 92 µg/mL, 107 µg/mL and 64 µg/mL, 90 µg/mL in HT-29 and Caco-2 cell lines at 48 h, respectively (Fig. 2). Also, treatment of KDR/293 normal cells (control group) with the highest determined concentration (140 µg/mL) at 24 h and 48 h time points didn't show cytotoxic effects. As shown in Fig. 2 and Fig. 3, beetroot extract and betanin significantly inhibited the growth of HT-29 and Caco-2 cell lines in a time and dose-dependent manner (with increasing concentrations from 40 to 100 µg/mL for betanin and 60 to 100 µg/mL for beetroot extract). Also, the lowest concentration amounts that significantly inhibited the growth rate of cancerous cells were determined as 60 µg/mL and 100 µg/mL for betanin and beetroot extract at 48 h respectively ( $p \leq 0.01$ ). Moreover, treatment with a higher concentration of beetroot extract increased the survival rates of HT-29 (at concentrations more than 100 µg/mL) and Caco-2 (at concentrations more than 120 µg/mL) cell lines at 48 h.

## Qualitative apoptosis assessment (DAPI staining)

Different apoptosis symptoms in nuclei and membrane of the cells were observed after treatment with beetroot extract, betanin, and 5-FU as the positive control group. As shown in Fig. 4, HT-29 and Caco-2 cells that treated with beetroot extract (92 µg/mL for HT-29 and 107 µg/mL for Caco-2), betanin (64 µg/mL for HT-29 and 90 µg/mL for Caco-2), and 5-FU (105 µL/well of 6-well plate) underwent condensed (early apoptosis) or fragmented (late apoptosis) nuclei and cell volume shrinkage, whereas whole control cells appeared as intact nuclei and membrane and were in a normal state.

## Quantitative apoptosis assay (flow cytometry)

Treatment with beetroot extract and betanin significantly increased percentage of cells in early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptosis in HT-29 and Caco-2 cancer cell lines after 48 h compared with the untreated control and normal KDR/293 cells that showed less cell death (Fig. 5). The total percentages of early and late apoptosis ratio after treatment with beetroot extract and betanin for 48 hours in the HT-29 and Caco-2 cell lines were found to be 81.7%, 91%, and 68.2%, 72.1% respectively. Also, in the positive control group (KDR/293 normal cells), the apoptosis ratio were determined as 21.5% and 38% respectively. Based on these findings, it seems that the apoptosis-inducing effect of betanin in cancerous and normal cell lines was more than beetroot extract. However, the apoptotic effects of betanin and beetroot extract on HT-29 and Caco-2 cell lines were comparable with 5-FU as an approved anticancer drug and these effects in KDR/293 cells were less than 5-FU.

## Gene expression levels

As shown in Fig. 6, treatment of HT-29 and Caco-2 cell lines with beetroot extract and betanin for 48 hours resulted in up-regulation of proapoptotic genes such as BAD, Fas-R, Caspase-3, Caspase-8, and Caspase-9. On the other hand, the expression level of the anti-apoptotic gene Bcl-2 was decreased significantly in both cancer cell lines after treatment with beetroot extract. Interestingly, the expression

level of proapoptotic gene BAD, in the HT-29 and Caco-2 cancer cells after treatment with beetroot extract and betanin was even higher than 5-FU as a positive control group. Moreover, the upregulation level of proapoptotic genes, Fas-R, Caspase-3, Caspase-8, and Caspase-9 were comparable with 5-FU.

Our findings showed that beetroot extract and betanin stimulated the intrinsic and extrinsic apoptosis pathways in treated HT-29 and Caco-2 cancer cell lines via downregulation of anti-apoptotic gene (Bcl-2) and upregulation of pro-apoptotic genes like BAD, Caspase-9, Fas-R, Caspase-3, and Caspase-8. In addition, the expression level of apoptotic genes in KDR/293 cells did not significantly change after treatment with beetroot extract and betanin.

## Discussion

Cancer is one of the leading causes of human death worldwide (37). Different factors that can decrease the incidence of chronic diseases could contribute to a significant increase in health and longevity. According to numerous reliable studies from the Europe and USA, higher consumption of fruits and vegetables was associated with a lower risk of total mortality (38, 39). In this regard, there has been growing epidemiological and experimental studies that suggest vegetables and fruits intake has an inverse relationship with chronic diseases such as cancer (37, 40). Recently, betalains as natural antioxidants with free radical scavenging and potential health benefits have been considered by supplements manufacturers (22, 41). Some studies have shown that the extract of red beetroot (*Beta vulgaris* L.) as an FDA approved red food color E162, can reduce the incidence of experimental tumors in skin, lung, liver, and esophagus in-vivo, and also is considered as a new natural product with potential chemopreventive and chemotherapeutic activities against human cancers (25, 27, 28). Despite several investigations related to the anticancer effects of beetroot and betanin, there are few studies about their anticancer activities and molecular pathways in colorectal cancer. Thus, we studied the anticancer activity of beetroot and betanin and also related molecular pathways in colorectal cancer cell lines. Our findings showed that beetroot extract and betanin can reduce cell proliferation (in different doses) in colorectal cancer cell lines via induction of apoptosis by modification of some key genes. On the other hand, KDR/293 cells, as the normal control group, treated with similar doses of beetroot extract and betanin remained intact after treatment at a similar time point.

In our study, beetroot extract inhibited cell proliferation in HT-29 and Caco-2 cell lines by a dose of 92 µg/mL and 107 µg/mL at 48 h time-point respectively. Besides, betanin, triggered cell apoptosis with lower doses than beetroot extract in HT-29 (64 µg/mL) and Caco-2 (90 µg/mL) cell lines without considerable apoptotic effects on normal KDR/293 cells.

Previous investigations showed that red beetroot extract (*Beta vulgaris*. L) has effective chemopreventive activity and can decrease cell proliferation, angiogenesis, inflammation and also can induce apoptosis in different cancer cell lines (26, 28, 30, 42). Likewise, various in-vitro and in-vivo studies suggested that betacyanins and isobetanin, reduce cancer cell proliferation with different IC50s and possess anti-inflammatory, hepatoprotective, radioprotective, hypolipidemic, and anti-diabetic effects in different doses



and time-points (30, 43). Additionally, the antiproliferative effects of red beetroot extract has been proven on androgen-independent human prostate (PC-3) and breast cancer cells (MCF-7) without considerable adverse effects on normal cells (26).

Betanin that makes more than 95% of the total betacyanins (300–600 mg/kg) is nontoxic in different concentrations on human umbilical vein endothelial cells (HUVECs) and normal human fibroblast cells and also inhibits ROS production, decreases intracellular ROS level about 3 folds (16, 17, 44), and enhances the caspase-3 activity in stimulated neutrophils within range of 100–300 mM (42, 44, 45). Our findings showed that red beetroot extract and betanin can induce apoptosis pathways (intrinsic and extrinsic) through downregulation of anti-apoptotic gene, Bcl-2, and upregulation of pro-apoptotic genes BAD, Fas-R, Caspase-3, Caspase-8, and Caspase-9. Likewise, treatment of MCF-7 cells with betanin increased the expression level of apoptosis-related proteins (BAD, TRAILR4, FAS, p-53), and altered the mitochondrial membrane potential (42). In another in-vitro study, Sreekanth et al. showed that betanin that isolated from the fruits of *Opuntia ficus-indica* decreases cell proliferation of human chronic myeloid leukemia cell line (K-562) with an IC<sub>50</sub> of 40 μM. Also, betanin induced intrinsic apoptosis pathway that is mediated by the release of cytochrome c from mitochondria into the cytosol, poly (ADP) ribose polymerase (PARP), downregulation of Bcl-2, reduction in membrane potentials and qualitatively causing chromatin condensation, cell shrinkage, and membrane blebbing (30). Despite the effective antiproliferative and apoptotic activities of beetroot extract, studies showed that it has lower cytotoxicity on normal cells in comparison with doxorubicin (Adriamycin) as a familiar chemotherapeutic agent (26, 46). In the same way, our results didn't show any apoptotic and cytotoxic effects on normal KDR/293 cells after treatment with beetroot extract and betanin at the same doses and time point.

Besides, several in-vivo studies reported that red beetroot and betanin significantly decreased tumor multiplicity (20%) and tumor load in the female A/J mice lung cancer model. Accordingly, betanin in the drinking water of mice lung cancer model inhibited the angiogenesis and increased the expression level of caspase-3. Besides, betanin triggered apoptosis via activation of caspase-3, -7, -9, and PARP in human lung cancer cell lines (24). In another study, Lechner et al. showed that regular oral consumption of red beetroot food color (78 μg/mL) decreased the number of NMBA-induced esophageal papilloma tumors by 45% and reduced cell proliferation in precancerous esophageal lesions in rats. Also, the level of angiogenesis and inflammation in the beetroot color-consuming rats were decreased, and the apoptotic rates were increased significantly (25). These results, along with our findings, confirmed that the anticancer effects of beetroot extract and betanin may be performed by induction of apoptosis in cancer cell lines. It seems that due to the local contact of colon cancer cells with beetroot extract and betanin in the intestinal tract circumstances, the antiproliferative and apoptotic effects of these compounds may be more effective than other types of cancers, which indicates the importance of using these natural compounds in the prevention and even treatment of this type of cancer.

Apoptosis as an accurate programmed cell death removes damaged cells via precisely regulated genes and plays an important role in the development and homeostasis of normal tissues (47, 48). A minimum defect in the apoptosis system can cause cancer or autoimmune diseases and its overactivation in some

cases may be the reason for degenerative diseases (49). Suppression of apoptosis in the carcinogenesis process is supposed to play a crucial role in the development and progression of some cancers (50). This exact mechanism is induced in malignant cells via either the caspase-mediated extrinsic or mitochondrial intrinsic pathways. Activation of effector caspases in both pathways resulting in morphological and biochemical cellular alterations like membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay (47, 51). However, the significant role of pro/anti-apoptotic proteins and over/down expression of their effective genes by natural anticancer compounds is very important in cell survival and apoptosis (32, 33, 52–54). During the process of preventing cancer cell formation, the DNA damage in precancerous lesions triggers apoptosis pathways with the purpose of removing potentially harmful cells and blocking tumor growth. Nevertheless, deregulation of this exact death process by different carcinogenic factors resulted in uncontrolled cell proliferation, progress, and development of cancerous cells and also predisposed to resistance against drug therapies (55, 56).

Although the beneficial effects of red beetroot and betanin on different cancers have been proven by several studies, the precise mechanisms of these effects are still unclear. Some of the hypotheses about the mechanism for chemotherapeutic and antiproliferative activities of betacyanins and betanin may be related to their ROS-reducing (reactive oxygen species) effects to a minimum level that stimulation for cell proliferation by inappropriate signal transduction couldn't happen at this level (57).

In the present study, we focused on betanin as the main beetroot betacyanin (up to 95%) that is the leading candidate for anticancer activity of red beetroot extract. Based on our qualitative (DAPI staining) and quantitative (flow cytometry) apoptosis assays results, red beetroot extract and betanin induced apoptosis pathways in both colorectal cancer cell lines, and this effect are comparable with a routine anticancer drug, 5-FU. Further studies including in-vivo models and clinical trials are needed to elucidate the exact cytotoxic and antiproliferative mechanisms of red beetroot extract and its main constituent, betanin or other effective compounds with anticancer activities in different cancers.

## Conclusion

In conclusion, the results of the present study showed that treatment of colorectal cancer cell lines (HT-29 and Caco-2) with red beetroot hydro-alcoholic extract and betanin inhibited cell proliferation and significantly induced apoptosis with low significant adverse effects on normal cells. Also, pro-apoptotic effects of beetroot hydro-alcoholic extract and betanin were performed via activation of intrinsic and extrinsic apoptosis pathways by downregulation or upregulation of main key genes. However, the exact antiproliferative and apoptotic mechanisms of beetroot extract and betanin on different cancer cells are still unknown and further studies are needed in this regard.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

AS and NA designed the research, developed the research plan, performed the experiments and statistical analyses, interpreted the data, and wrote the manuscript. MHS and AYK reviewed the paper and provided comments. All authors read and approved the final manuscript.

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## Figures

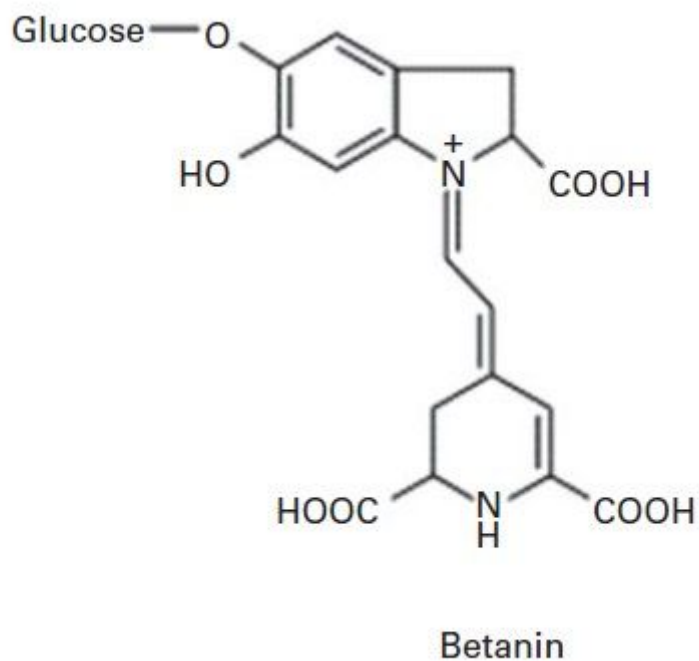
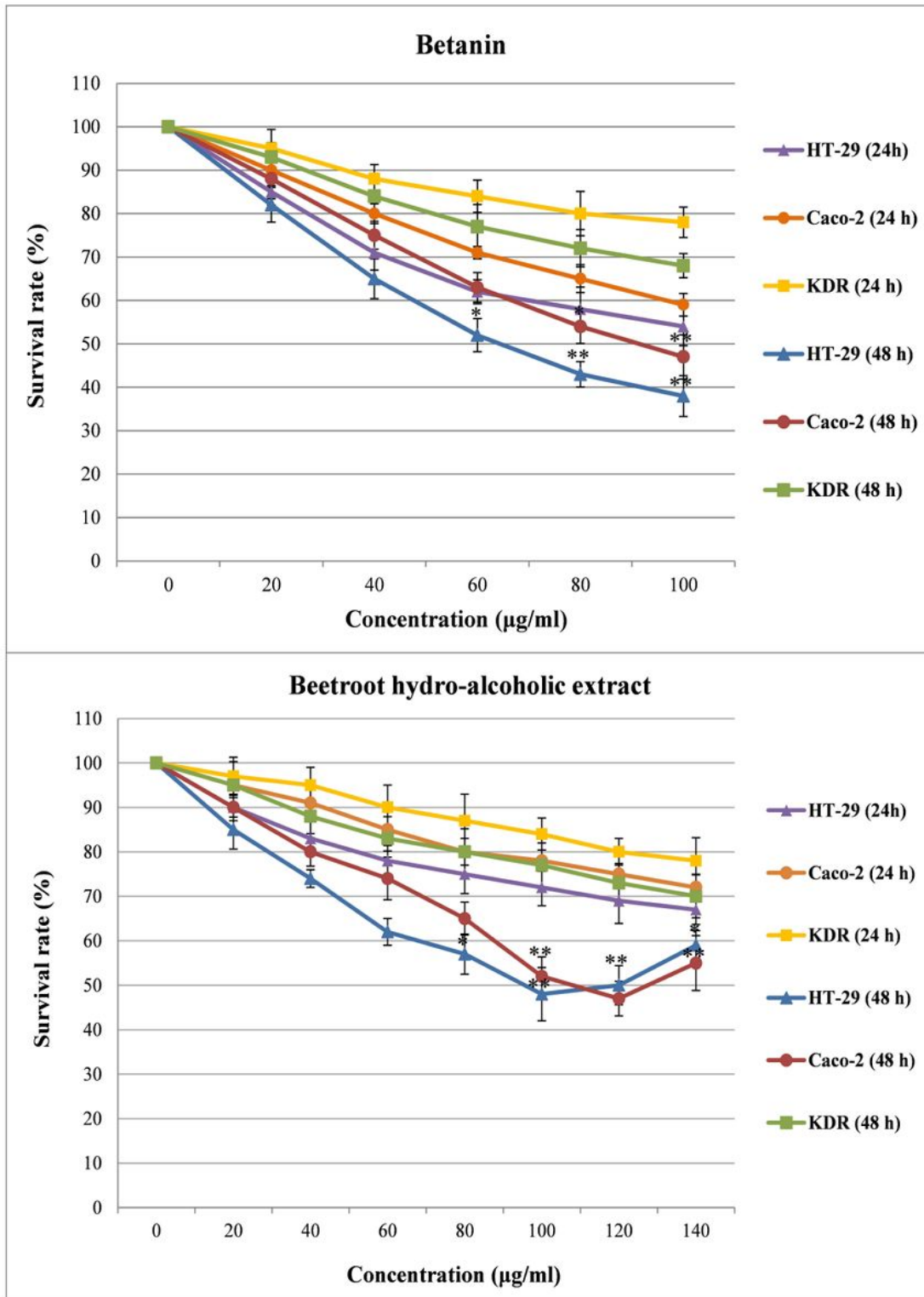


Figure 1

General chemical structure of Betanin (betanidin-5-O-b-glycoside) (18)

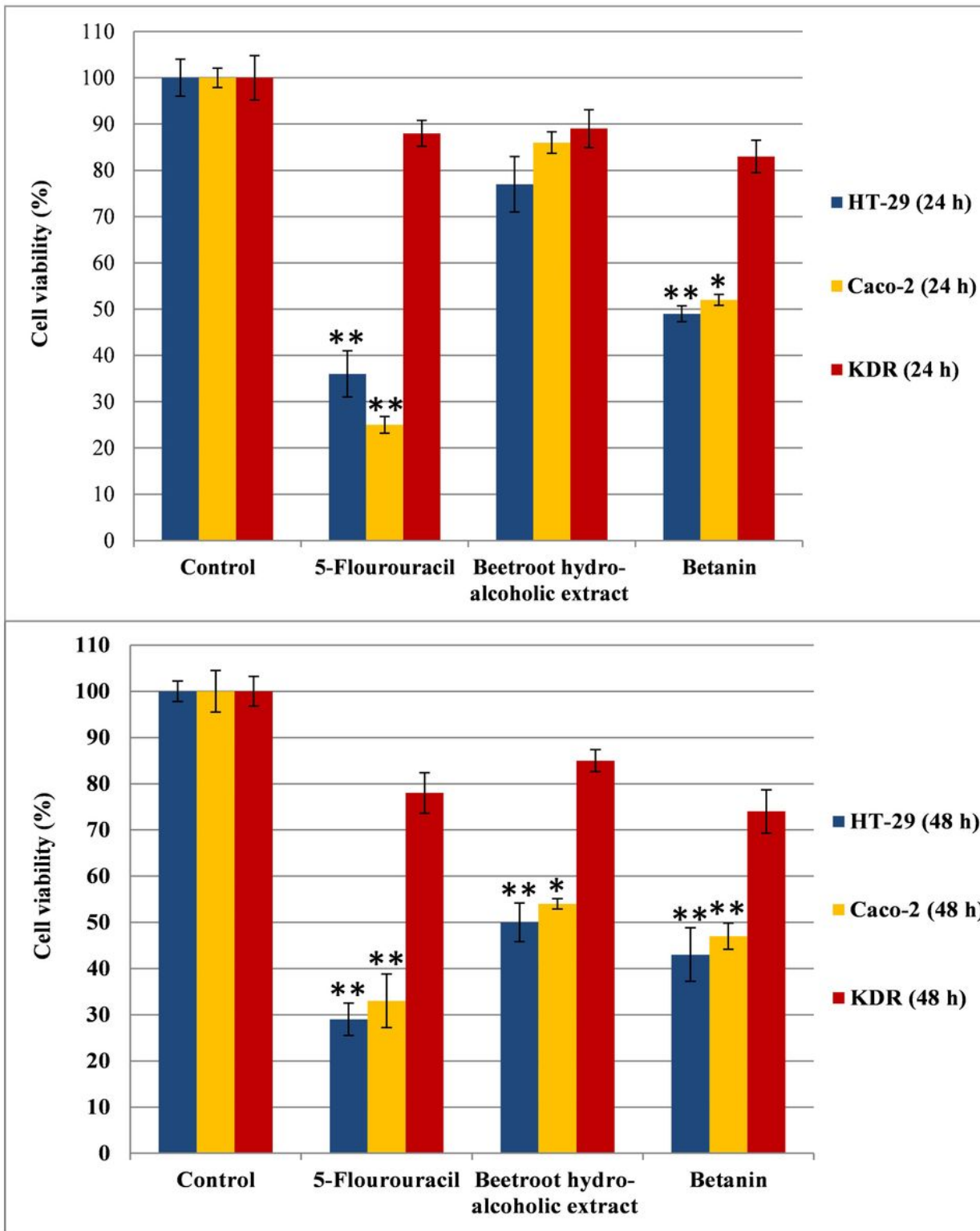


**Figure 2**

MTT assays were used to determine half maximal inhibitory concentration (IC<sub>50</sub>) in HT-29 and Caco-2 cells after treatment with different concentrations of betanin and beetroot extract (in the range of 20 to 140 µg/ml) for 24 and 48 h. The effective doses for beetroot extract were determined about 92 µg/mL, 107 µg/mL and for betanin about 64 µg/mL, 90 µg/mL for HT-29 and Caco-2 cells at 48 hours, respectively. Treatment with betanin significantly decreased survival rate in HT-29 and Caco-2 cells while



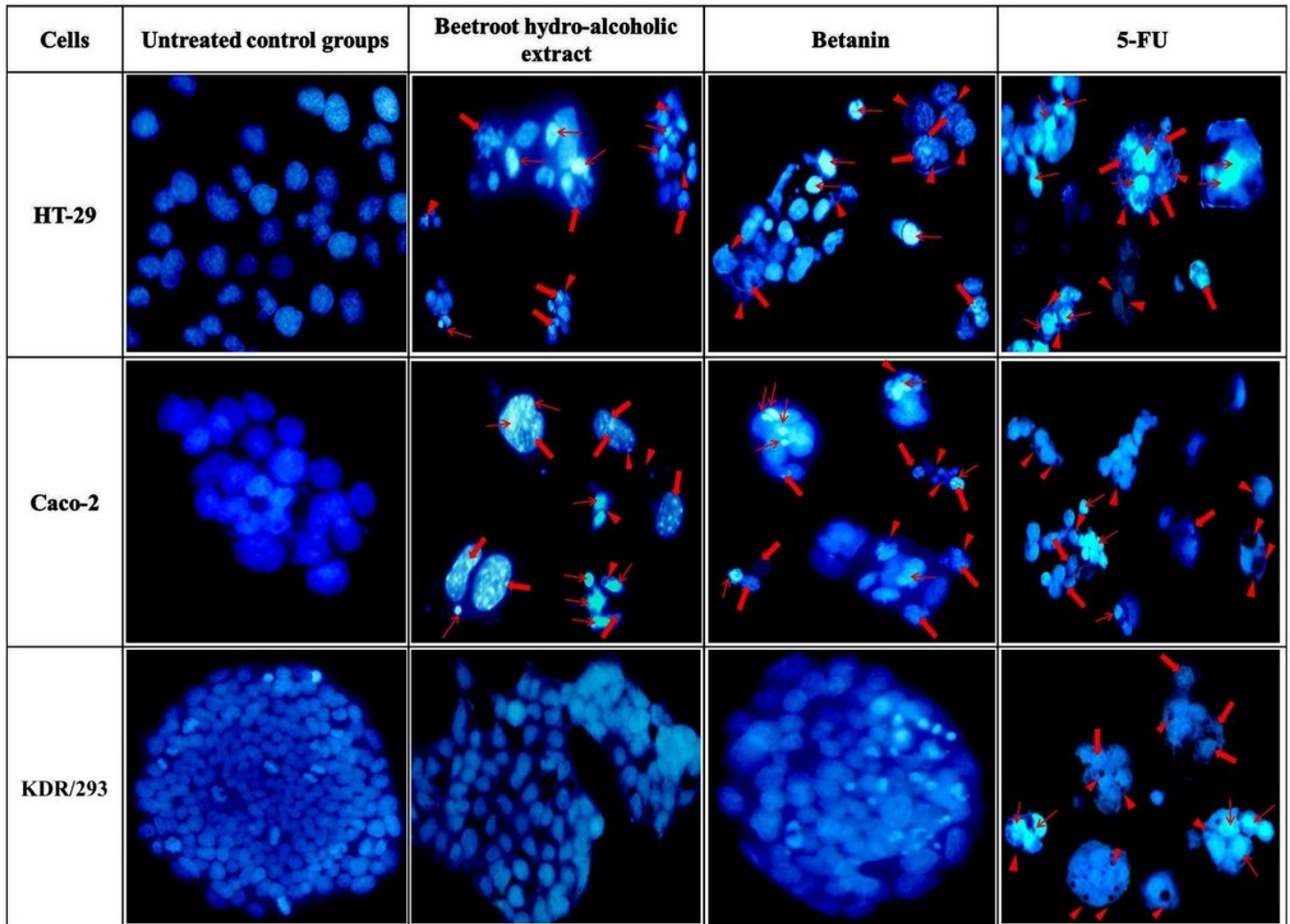
KDR/293 normal cells remained intact. Data were obtained from 3 independent experiments for each test and were normalized to naive cells and presented as percent viable cells (means  $\pm$  SD). Asterisks indicate a significant increase in cell death (\*\* $p \leq 0.01$ , \* $P < 0.05$ ).



**Figure 3**

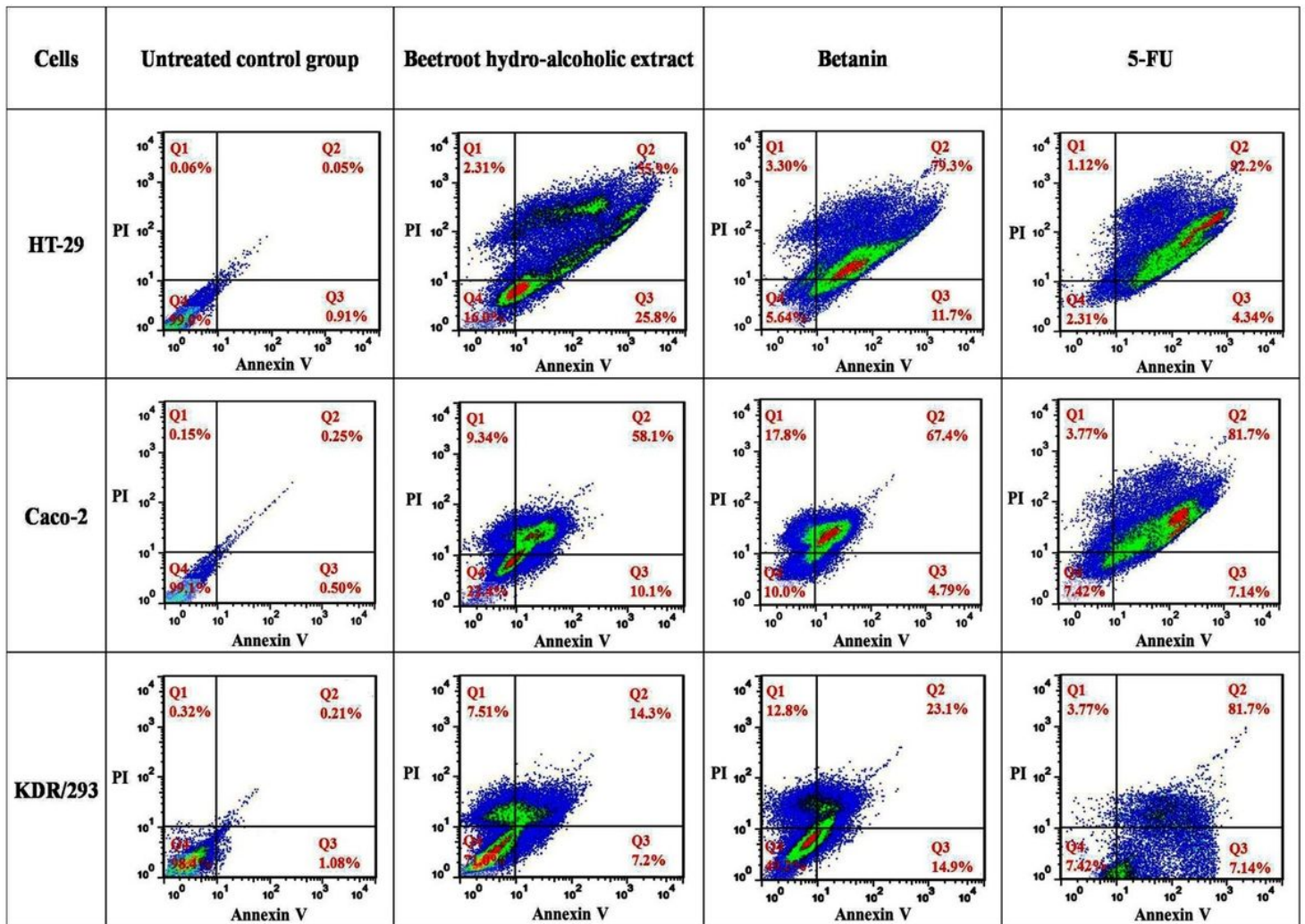
Effect of beetroot extract and Betanin on the viability of HT-29, Caco-2 and KDR/293 cell lines. Cell viability was expressed as the percentage of optical density of the treated cells in comparison with

untreated controls (100% viability). All the experiments were performed in triplicate (n = 3), and the data were presented as means  $\pm$  SD. \*P  $\leq$  .05 and \*\*P  $\leq$  .01 indicate significant and highly significant vs the control group.



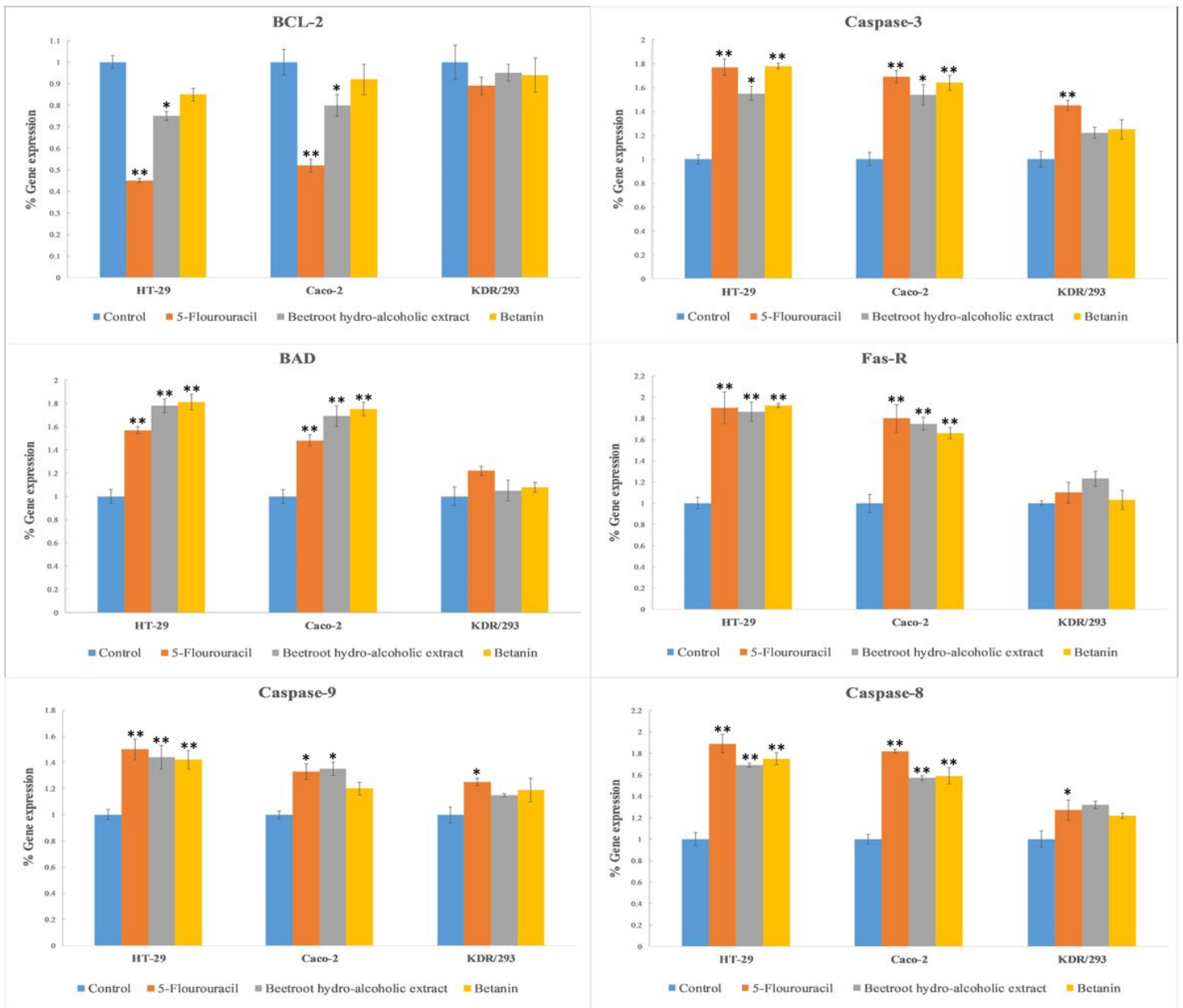
**Figure 4**

DAPI staining of treated/untreated HT-29T, Caco-2 and KDR/293 cells. Panels represent untreated control groups, treated with beetroot extract, treated with Betanin and treated with 5-FU (105  $\mu$ l/well) as positive control groups for 48 h incubation, respectively. Arrows depict chromatin condensation (thin arrows), fragmented nuclei (thick arrows) and membrane blebbing (arrowheads).



**Figure 5**

Flow cytometry analysis of treated/untreated cancerous and normal cells. Cells were treated with FITC-Annexin V in combination with PI to detect apoptosis and necrosis before being subjected for analysis by flow cytometry. Panels represent untreated control groups, treated with beetroot extract, treated with betanin and treated with 5-FU (105  $\mu$ L/well of 6-well plate) as positive control groups for 48 h incubation, respectively. Dots with Annexin V-/PI+ (Q1), Annexin V+/PI+ (Q2), Annexin V+/PI- (Q3), and Annexin V-/PI- (Q4) and feature represent necrotic, late apoptotic, early apoptotic, and viable intact cells, respectively.



**Figure 6**

Expression level of key apoptosis pathway genes in the HT-29, Caco-2, and KDR/293 cancer cells lines after treatment with beetroot extract, betanin, and 5-FU (105  $\mu$ L/well of 6-well plate) as positive control group for 48 h incubation. Target genes were normalized to GAPDH as housekeeping control gene (\* $p \leq 0.05$ , \*\* $p \leq 0.01$  as compared to control).