Anticancer Effects of Beetroot Hydro-Alcoholic Extract and Betanin on Human Colorectal Cancer Cell Lines

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Abstract

Background: 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: Hydro-alcoholic extract of red beetroot 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 (IC₅₀) 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 time and doses.

Results: The IC₅₀ 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 hydro-alcoholic 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.

Conclusion: Betanin effectively inhibited cell growth in both colorectal cancer cell lines with no significant cytotoxic effects on the KDR/293 normal cells. The mechanism of the anticancer effects of red beetroot hydro-alcoholic extract and betanin needs to be further studied.

Background

Colorectal cancer (CRC) has known as the main cause of cancer-related death worldwide and leading to 693,900 deaths in both women and men in 2012.[1, 2] Besides, 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 are rising among young adults.[3, 4] 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 family history, inflammatory bowel disease, smoking, excessive alcohol consumption, high consumption of red and processed meat, obesity and diabetes have been identified.[4, 5] Thus, 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.[6–9] Several studies have demonstrated a reverse relevance between consumption of fruits and vegetables with the risk of chronic diseases and malignancies.[10–13] Nowadays, fruits and vegetables as rich sources of bioactive compounds with high antioxidant capacity attracted much attention in human nutrition and medicine. However, well-known dietary antioxidants such as ascorbic acid, R-tocopherol, carotenoids, anthocyanins (flavonoid glycosides) and recently the betalains [14–16] have been targeted by different investigators due to potential capacity to scavenge free radicals, preventive effects on active

oxygen-induced and free radical-mediated oxidation that causing different cellular damage.[15, 17, 18] Nevertheless, studies revealed that natural pigments of fruits, vegetables, green tea, and red wine exert significant health beneficial activities and antioxidant effect via their active phenolic compounds, flavonoids and other phytochemicals.[18, 19] The red-violet betacyanins (e.g., betanin and isobetanin) and the yellow betaxanthins (e.g., vulgaxanthin I and II) are two water-soluble nitrogenous pigments in members of most families of the plant order Caryophyllales (except the Caryophyllaceae and Molluginaceae) and in some higher fungi.[16, 20] Among the various natural sources of betalains, red and yellow beet, colored Swiss chard, grain amaranth, and cactus fruits are the only foods containing these compounds.[15, 21, 22] Red beetroot is a good source of potassium, calcium and magnesium salts, vitamin C, folic acid, and dietary fiber.[18, 23–26] Moreover, red beetroot (*Beta vulgaris* L.) belongs to the vegetables family that owing to high antioxidant capacity due to the presence of betalains that are composed of red pigments (betacyanins) and yellow pigments (betaxanthins) that collectively known as betalains.[27, 28] Betalains as the main constituent of red beets are consisting of mostly betanin, isobetanin, and vulgaxanthin I & II and used as natural colorant in numerous food industries such as dairy products (e.g. milk, ice creams, yogurt, and kefir), beverages, candies (e.g. cookies and desserts) and cattle products (cooked, smoked, semi-dry or fermented sausage).[29–32] Betanin as principal active phytochemical of beetroot is water-soluble nitrogenous compound and comprises 75–95% of red beetroot pigments (300–600 mg/kg) (Fig. 1).[18, 20, 27, 33] 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.[15, 16, 28, 34] It has been demonstrated that beetroot and its main constituent, betanin, can be effective as strong chemopreventive agent who induces apoptosis and decreases cell proliferation, angiogenesis, inflammation and stimulates apoptosis in skin, liver, lung, and esophageal cancer in experimental animals and cancer cell lines.[28, 35–38] Beetroot extract showed effective cancer chemopreventive activity and inhibit the 7, 12-imethylbenz[a]anthracene initiated tumorigenesis in mouse skin and diethylnitrosamine induced mouse hepatocarcinogenesis.[28, 39] It was shown also that treatment of human chronic myeloid leukemia cell line-K562 with 40 mM betanin decrease cell proliferation (50%) and induce the intrinsic apoptosis pathway. Beside, betanin treatment resulted in a time-dependent increase in the PARP cleavage in the K562 cell line and activated caspase-3 as an executioner caspase in apoptotic cascades.[40, 41] Furthermore, Lechner et al. showed that consumption of the red beetroot water for a period of 35 weeks reduced the levels of angiogenesis and inflammation and increased apoptosis in the beetroot consuming rats with esophagus tumor compared with a control group without any significant toxicity. Also, betanin is able to modulate ROS production and responsible for the effect of beetroot on neutrophil oxidative metabolism and inhibited DNA damage and apoptosis. [36] 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.[28, 36] Currently, despite numerous investigations, there is limited information about molecular mechanisms by which betanin exerts its anticancer effect. Moreover, the effects of red beetroot and betanin on colon cancer have not been widely explored. Thus, the aim of the

present study was to further explore and compare the mechanism of anticancer activity of hydro-alcoholic extract of beetroot and its major component; Betanin on colorectal cancer cell lines (Caco-2, HT-29) in comparison with normal epithelial cells (KDR).

Methods

Plant Material

Seeds of beetroot were sown in the experimental farm and the resulting plants were cultivated for. The collected 4 months of mature beetroots leaves were removed and were washed with water, then the cleaned roots were chopped and used for hydro-alcoholic extraction.

Beetroot hydro-alcoholic extraction

To prepare dry beetroot hydro-alcoholic extract, 1 kg of fresh biomass was homogenized via blender. The obtained liquid was used for extraction as follows: I) mixing the beetroot juice three times with 70% ethanol II) centrifuging the mixture at 10,000 g for 30 min and evaporating the supernatants at 40 °C under vacuum up to dry. The dried total extract was dissolved in 1000 mL of 70% methanol, subsequently were refrigerated at – 20 °C for 24 h, after that the supernatant was carefully isolated from the precipitate. The aqueous fractions that remained from the methanol-evaporated supernatants were lyophilized (Christ, Alpha 1–2, Germany) and used as dry beetroot extracts.

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 cm² 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% CO₂. 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 hydro-alcoholic extract, betanin (Sigma-Aldrich Chemie GmbH) and 5-Fluorouracil (5-FU) (7 µl/well) as described in our previous studies.^{65,66} The half-maximal inhibitory concentration (IC₅₀) was 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).

Morphological analysis

DAPI staining method was used for finding morphological changes regarding all untreated and treated groups with beetroot hydro-alcoholic extract, betanin, and 5-Fluorouracil (5-FU) as exactly described in our previous studies [42, 43].

Flow cytometry

Colorectal cancer cell lines (HT-29 and Caco-2) and normal KDR cells were seeded into a six-well culture plate (1.2×10^5 cells/well) and treated similar to DAPI staining method. After treatment time point, the treated/untreated cells were detached by Trypsin-EDTA (Sigma-Aldrich, St Louis, MO), and liquid phase including beetroot hydro-alcoholic extract and betanin were 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 dH₂O) and centrifuged and their supernatants were disposed of. Then, the flowcytometry assessments were performed on all untreated/treated cells as exactly described in our previous studies.^{65,66}

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

The IC₅₀s after treatment with different doses (20 to 140 µg/ml) in the two time-points (24 and 48 h) 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 in the HT-29 and Caco-2 cells at 48 hours, respectively (Fig. 2). Also, treatment of the KDR/293 normal cells (as a 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 hydro-alcoholic 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 hydro-alcoholic extract) and showed significant inhibition at concentrations of 100 µg/mL after betanin and beetroot hydro-alcoholic extract treatments for 48 h ($p \leq 0.01$). Moreover, treatment with a higher concentration of beetroot hydro-alcoholic extracts increased the survival rates of HT-29 (at a concentration more than 100 µg/mL) and Caco-2 (at a concentration more than 120 µg/mL) cancer cell lines at 48 h, respectively.

Morphological analysis of the cell lines

Different apoptosis symptoms in nuclei and membrane of the cells were observed after treatment with beetroot hydro-alcoholic extract, betanin, and 5-FU as a positive control group. As shown in Fig. 4 the HT-29 and Caco-2 cells that treated with beetroot hydro-alcoholic 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.

Flow cytometry

According to the flow cytometry results, treatment with beetroot hydro-alcoholic 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 hydro-alcoholic 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 about 21.5% and 38% respectively. Based on these findings the apoptosis-inducing effect of betanin in cancerous and normal cell lines were more than beetroot hydro-alcoholic extract. However, the apoptotic effects of betanin and beetroot hydro-alcoholic extract on HT-29 and Caco-2 cell lines were comparable with 5-FU as approved anticancer drug and these effects in KDR/293 cells were less than 5-FU.

Discussion

Cancer is one of the leading causes of human death worldwide.[44] 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 Europe and the USA, higher consumption of fruits and vegetables were associated with a lower risk of total mortality.[45, 46] 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.[44, 45, 47–49] Recently, betalains as natural antioxidants with free radical scavenging and potential health benefits have been considered by supplements manufacturers.[32, 50–52] Numerous investigations showed that the extract of red beetroot (*Beta vulgaris* L.), the FDA approved red food color E162, can reduce the incidence of some experimental tumors in skin, lung, liver and esophagus *in vivo* and is considered as new natural product with chemopreventive and/or chemotherapeutic activity opposing human cancers. [28, 36, 38] Likewise, our results showed that the beetroot hydro-alcoholic extract and its main constituent, betanin, can reduce the cell proliferation (in different doses) in the colorectal cancer cell lines via induction of apoptosis. Interestingly, the KDR/293 cells as a normal control group that treated with similar doses of beetroot hydro-alcoholic extract and betanin were intact in the determined treatment time point. The beetroot hydro-alcoholic extract inhibited cell proliferation in the HT-29 cells by a dose of 92 µg/mL, as well in the Caco-2 cells by a dose of 107 µg/mL at 48 h time-point. Also, betanin, triggered cell apoptosis with lower doses than beetroot hydro-alcoholic extract in HT-29 (64 µg/mL) and Caco-2 (90 µg/mL) cell lines without considerable adverse 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 such as Human lymphoma cells (Raji cells),[38] Human melanoma cells (B16F10),[53] Human chronic myeloid leukemia cells (K562),[40] Human colonic adenocarcinoma cells (HT-29), Human liver hepatoma cells (Huh7) [54] and Human breast cancer cell line (MCF-7).[37, 55] Indeed, various in-vitro and in-vivo investigations suggested that betacyanins, isobetanin and betanin as the main constituent of red beetroot, reduce cancer cell proliferation with different IC₅₀s and possess anti-inflammatory, hepatoprotective, radioprotective, neuroprotective, diuretic, hypolipidemic, osteoarthritis pain reliever and anti-diabetic effects in different doses and time-points.[40, 56] Moreover, the antiproliferative effects of red beetroot extract have been proven on androgen-independent human prostate (PC-3) and breast cancer cells (MCF-7) without considerable adverse effects on normal human skin (FC) and liver (HC) cells.[37] As well, betanin that makes more than 95% of the total betacyanins (300–600 mg/kg) and known as the most principal and effective compound of the red beetroot is nontoxic, in different concentrations, on human umbilical vein endothelial cells (HUVECs) and normal human fibroblasts cells and also significantly inhibits ROS production, decreases the intracellular ROS level about 3 fold,[18, 20, 57] enhances the caspase-3 activity in stimulated neutrophils with the doses within range of 100–300 mM.[55, 57, 58] Likewise, treatment of MCF-7 cells with betanin-enriched red beetroot (*Beta vulgaris* L.) extract increased the expression level of apoptosis-related proteins (Bad, TRAILR4, FAS, p53) and altered the mitochondrial membrane potential. These alterations confirm the involvement of both intrinsic and extrinsic apoptosis pathways due to red beetroot extract treatment.[55] However, beetroot extract showed lower cytotoxicity on normal cells in comparison with doxorubicin (Adriamycin) as familiar chemotherapeutic agent.[37, 59] In another in-vitro study, Sreekanth *et al.* showed that betanin, isolated from the fruits of *Opuntia ficus-indica* decreases cell proliferation of human chronic myeloid leukemia cell line (K562) with an IC₅₀ 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 the membrane potentials and qualitatively causing chromatin condensation, cell shrinkage and membrane blebbing.[40] Similarly, our investigation demonstrated that red beetroot hydro-alcoholic extract and betanin can inhibit cell proliferation and can induce apoptosis in treated HT-29 and Caco-2 cancer cell lines.

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 resulted in induction of apoptosis. Betanin also triggered apoptosis via activated caspase-3, -7, -9, and PARP in human lung cancer cell lines.[35] 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 both precancerous esophageal lesions and in papillomas of NMBA-treated rats. Also, the levels of angiogenesis and inflammation in the beetroot color-consuming rats were decreased, and the apoptotic rates were increased significantly.[36]

Although the beneficial effects of red beetroot and betanin on different cancers have been proven by numerous studies, the precise mechanisms of these effects are still unclear. Based on several in-vitro and in-vivo studies it can be hypothesized that the betacyanins and betanin possessed chemotherapeutic and

antiproliferative activities through their antioxidative activity and decrease the level of reactive oxygen species to minimum level that can't stimulate proliferation by inappropriate signal transduction in that levels.[60]

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.[61, 62] The least defect in this system can cause cancer or autoimmunity, while the improvement of apoptosis in some cases may cause degenerative diseases.[63] Indeed, suppression of apoptosis in the carcinogenesis process is supposed to play a crucial role in the development and progression of some cancers.[64] This exact mechanism 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.[61, 65] 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 the cell survival and apoptosis.[42, 43, 66–68] 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.[69, 70]

Thus, the previous studies suggested that the betalains are the most principal and potent anticancer constituents of the red beetroot extract.[18, 36, 71–73]

Conclusion

In the present study, we focused on betanin as the major (up to 95%) beetroot betacyanins that are the leading candidate for anticancer activity of red beetroot extract. According to our results based on qualitative (DAPI staining) and quantitative (flowcytometry) apoptosis assays, red beetroot extract, and betanin, with different doses, induced apoptosis pathways in both colorectal cancer cell lines and this effects 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. In conclusion, the present study indicates that treatment of colorectal cancer cell lines (HT-29 and Caco-2) with red beetroot hydro-alcoholic extract and betanin inhibited the cell proliferation and significantly induced apoptosis with low significant adverse effects on normal cells. However; the exact antiproliferative and apoptotic mechanisms of beetroot extract and betanin on different cancer cells is still unknown and further studies are needed in this regard.

Abbreviations

CRC: Colorectal cancer

PARP: Poly ADP ribose polymerase

ROS: Reactive oxygen species

RPMT: Roswell Park Memorial Institute medium

DMEM: Dulbecco's Modified Eagle's Medium

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ELISA: Enzyme-linked immunosorbent assay

DAPI:4', 6-diamidino-2-phenylindole

HUVEC: Human umbilical vein endothelial cells

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

Funding

None.

Authors' contributions

Amir saber, Nasim Abedimanesh designed the study and collected the data. Amir Saber, Ahmad Yari Khosroushahi carried out data analyses and produced the initial draft of the manuscript. Mohammad-Hossein Somi revised the manuscript critically. 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). [27]

Figure 2

MTT assays were used to determine half maximal inhibitory concentration (IC₅₀) in HT-29 and Caco-2 cells after treatment with different concentrations of betanin and beetroot hydro-alcoholic extract (in the range of 20 to 140 µg/ml) for 24 and 48 h. The effective doses for beetroot hydro-alcoholic 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 hydro-alcoholic 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 ± SD. *P ≤ .05 and **P ≤ .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 hydro-alcoholic extract, treated with Betanin and treated with 5-FU (105 µl/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 cytometric 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 hydro-alcoholic extract, treated with betanin and treated with 5-FU (105 μ 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.