

Article

Young Shoots and Mature Red Cabbage Inhibit Proliferation and Induce Apoptosis of Prostate Cancer Cell Lines

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Abstract: Prostate cancer is one of the most common cancers in men. Recent dietary and epidemiological studies have suggested the benefit of dietary intake of cruciferous vegetables in lowering the incidence of cancer. The health promoting effects of red cabbage are attributed to their mixture of phytochemicals known for their antioxidant and anticancer activity. In the current study, we investigated whether young shoots and mature red cabbage had any effect on prostate cancer cell lines (DU145 and LNCaP). Attempts were also made to identify the potential molecular mechanism(s) by which plant material elicits its biological effects on prostate cancer cell lines. Here we report that the studied vegetable inhibited the proliferation of cancer cells and that this process was associated with the induction of apoptosis via caspase-dependent and both extrinsic and intrinsic pathways. In addition, we also observed the regulation of genes and proteins associated with cell survival and apoptotic events.

Keywords: young shoots; red cabbage; apoptosis; prostate cancer cell lines



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1. Introduction

Cancer is a leading cause of death globally and the most common are breast, lung, colon and rectum, prostate, and skin cancers [1]. Consequently, in order to reduce the risk of cancer, doctors recommend paying attention to nutrition, body weight, physical activity, and lifestyle. Thus, it is reasonable to search for and implement effective anti-cancer prevention methods. Numerous researches confirm the positive impact of bioactive plant-derived food ingredients on the risk of diseases, including cancer [2]. Potential mechanisms for chemopreventive properties of phytochemicals, include enhanced carcinogen elimination, inhibition of inflammatory processes and tumor angiogenesis, as well as a direct cytotoxic effect on tumor cells [3].

Among vegetable classes, strong associations with reduced cancer incidence have been found for Cruciferae vegetables. It is the family of plants that includes various similar members of the Brassica oleracea species that are widely consumed in various parts of the world. There is increasing evidence that one of the members of crucifers, red headed cabbage (*Brassica oleracea* var *capitata* f. *rubra*), plays a special role in protection against cancer due to their unique chemical content, most notably their high levels of glucosinolates, polyphenols, and vitamins. In addition, red cabbage is low in fat, especially saturated fat, high in fiber, and contains many minerals [4]. Glucosinolates are the main class of specialized metabolites found in cruciferous plants and are responsible for the chemopreventive activity of these vegetables. Glucosinolates breakdown products modulate the action of enzymes of phase I and II detoxification associated with the elimination of xenobiotics from the body. Moreover, these compounds have been shown to arrest cell cycle, inhibit growth, and induce apoptosis in various cancer cell lines in vitro [5]. Apoptosis is programmed cell death, which can be induced either by an intrinsic mitochondria-mediated pathway or by

an extrinsic pathway involving the activation of the TNF/Fas death receptor family. Both pathways lead to the activation of caspases, the major executioners of this process, which are responsible for the execution of cell death [6].

Edible sprouts and young shoots of cabbage represent a valuable source of diverse macro- and micronutrients and healthy secondary metabolites [7]. Some studies found that sprouts possess higher nutritional value in comparison to their mature counterparts [8,9]. It is widely known that the consumption of cruciferous sprouts prevents health problems, based on the presence of a number of bioactive secondary phytochemicals, which have the capacity to act on diverse molecular targets in cells.

In our previous studies, we observed that young shoots of red cabbage in the phase of intensive growth are richer in glucosinolates, vitamin C, carotenoids, and some minerals and were also characterized by a higher antioxidant activity than the mature vegetable [10,11]. In addition, the juice of young shoots has a higher content of vitamin C than the juice of the mature vegetable and a similar amount of polyphenolic compounds. Plant materials were used to prepare juices, which is described in the current manuscript. The study examines, possibly for the first time, the potential *in vitro* antitumor activity of fresh, and primarily *in vitro* digested and absorbed, juice of young shoots and of its mature vegetable, by using the approach of molecular methodologies. It aims to explore the possible mechanisms used by the juices to inhibit the proliferation of human prostate cancer cell lines (DU145 and LNCaP). The current study was also designed to determine whether juices had the potential to induce apoptosis in cancer cells using three techniques: Flow cytometry, RT qPCR, and Western blot analysis, which together detect and measure factors involved in the process of cell death.

2. Materials and Methods

2.1. Plant Material

The analyzed material was 14-day young shoots of red headed cabbage and the mature vegetable (polish variety Haco). The growing conditions of cabbage were described in our previous publication [10]. The fresh material (about 200 g of shoots and 300 g of cutting different-sized heads of cabbage) was used to prepare juices (by squeezing in a juicer). The samples were closed in plastic tubes to protect them from light and stored in a freezer (20 °C).

2.2. Simulation *In Vitro* Digestion Model of the Gastrointestinal Tract

Digestion of the juice of young shoots and of mature red cabbage was performed *in vitro* in two steps according to Minekus et al. [12]. In order to create conditions reflecting the *in vivo* processes occurring in the stomach, pepsin (Sigma-Aldrich, ST. Louis, MO, USA) was used and pH 2.0 was adjusted (the mixture was incubated for 2 h at 37 °C, with shaking). In order to make the environment similar to that of the intestine, pancreatin from porcine pancreas (Sigma-Aldrich) and bile salts (Sigma-Aldrich) was added, the pH to 7.4 was increased (the mixture was incubated for 2 h at 37 °C, with shaking). The next step was the introduction of intestinal microflora Leibniz Institut DSMZ, Braunschweig, Germany) and incubation for 16 h at 37 °C [13].

2.3. Simulation *In Vitro* Absorption Model of the Gastrointestinal Tract

The digested *in vitro* product was sterilized by filtration through nitrocellulose filters. A human colon cancer cell line Caco-2 was used to study the transport of digestive products. Culture of Caco-2 cells leads to the establishment of monolayers. Cells were harvested at 90% confluence with trypsin-EDTA and seeded onto PET inserts (BIOKOM, Janki, Polska), mounted in 12-well plates, at a total density of 1×10^5 cells per well. The culture medium was replaced every other day and the cell's monolayer was grown for 21 days. Caco-2 monolayers confluence was examined by measuring transepithelial electrical resistance (TEER). Values of TEER higher than $200 \Omega/\text{cm}^2$ were used for permeability experiments. Caco-2 monolayer was washed twice with PBS solution (Sigma-Aldrich). Then, the digested

juices were placed on the Caco-2 cells culture. After 2 h of incubation, the filtrate was collected and secured for subsequent analysis.

2.4. Cell Culture

The human prostate carcinoma DU145 (not detectably hormone sensitive, ATCC[®] HTB-81[™]), LNCaP cell lines (androgen receptor, positive; estrogen receptor, positive; ATCC[®] CRL-1740[™]), and the human colon cancer cell line Caco-2 were purchased from the American Type Culture Collections (ATCC, Manassas, VA, USA). The human normal prostate PNT-2 cell line was purchased from HPA Culture Collections (Sigma-Aldrich). Cells were cultured in RPMI 1640 or MEM medium (Sigma-Aldrich) according to the ATCC instruction with an addition of 10% FBS (Sigma-Aldrich).

2.5. Cell Treatment

For cytotoxicity and cell proliferation assessment, DU145, LNCaP, and PNT-2 cell lines were seeded on 96-well plates (8×10^4 cells per well). A total 24 h after seeding, the growth medium was replaced with filtrates (juices subjected to in vitro gastrointestinal digestion and absorption) for 24 h of incubation. For Muse[®] flow cytometer, RT-qPCR and Western blot analysis, and DU145 and LNCaP cell lines were seeded on 6-well plates (2×10^5 cells per well). A total 24 h after seeding of cells, the growth medium was replaced with whole filtrates with no medium added (digested and absorbed juices), as well as with fresh 5% juices (prepared in appropriate culture medium), for 24 h of incubation. Untreated cells, grown in the standard culture medium, without any juices, were used as a control of all experiments. Staurosporine (a final concentration of 1.5 μ M) was used as a positive control of the activation of apoptotic proteins, the process of apoptosis, and as a confirmation of the correctness of the assumed experimental conditions.

2.6. Cytotoxicity Analysis

The cytotoxicity assessment of the studied juices was performed by Cytotoxicity Detection Kit LDH (Sigma-Aldrich) according to the manufacturer's instruction. Each treatment included 3 biological and 3 technical replicates.

2.7. Cell Proliferation Assay

Cell proliferation was analyzed using the 5'-bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit III (Sigma-Aldrich) according to the instruction provided by the manufacturer. Each treatment included 3 biological and 3 technical replicates.

2.8. The Muse[®] Flow Cytometer Analysis

The cells were labeled following the manufacturer's protocol for the analyses: The Muse[®] Annexin V & Dead Cell Assay Kit, Muse[®] MultiCaspase Assay Kit, and Muse[®] Bcl-2 Activation Dual Detection Assay Kit (Merck, Kenilworth, NJ, USA). Analyses were performed using a Muse[®] Cell Analyzer (Merck).

2.9. RNA Isolation, cDNA Synthesis and RT-qPCR Analysis

Total RNA from cancer cell cultures was isolated with the use of Total RNA Mini Plus Kit (A&A Biotechnology, Gdynia, Poland), following the manufacturer's instruction. Reverse transcription of total RNA to the cDNA was performed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). A quantitative verification of genes was performed using CFX96 Touch[™] Real-Time PCR Detection System instrument (Bio-Rad). The PCR reaction mixture contained cDNA samples, RNase-free water, SYBR Green Supermix (Bio-Rad), and primers for following genes: Apoptosis-inducing factor mitochondria associated 1 (*AIFM1*), AKT serine/threonine kinase 1 (*AKT1*), apoptotic peptidase activating factor 1 (*Apaf-1*), Bcl2-associated agonist of cell death (*BAD*), Bcl2-binding component 3 (*BBC3*), caspase-3 (*CASP-3*), caspase-7 (*CASP-7*), caspase-8 (*CASP-8*), diablo IAP-binding mitochondrial protein (*DIABLO*), Fas-associated death domain (*FADD*),

Fas cell surface death receptor (*FAS*), and tumor protein p53 (*TP53*). The conditions of individual PCR reactions were optimized for the given pair of oligonucleotide primers (Table S1). Basic conditions were as follows: 95 °C for 2 min (initial step), 45 PCR cycles at 95 °C for 15 s (denaturation) and 60 °C for 30 s (annealing), and 75 °C for 1 min (extension). Results were normalized using reference gene β -actin (*ACTB*) and were determined using the $2^{-\Delta\Delta CT}$ method as previously reported by Livak and Schmittgen [14].

2.10. Western Blot Assays

Cell lysis was carried out with use Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of Protease Inhibitor Cocktail (BioShop, Burlington, ON, Canada) according to the protocol provided by the manufacturer. The PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) test was used to determine the protein content in the cell lysate, and the analysis itself was carried out using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific). To investigate the mechanism of action of the experimental juices, proteins of the cell lysate were separated using Mini-PROTEAN TGX Stain-Free precast gels (Bio-Rad). Next, the Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-Rad) were used, for the fast, efficient transfer of proteins from gels using the Trans-Blot[®] TurboTM Transfer System (Bio-Rad). Subsequently, the immobilized proteins were incubated with the appropriate primary antibody: Apoptotic protease-activating factor 1 Apaf-1 (#8723), Bcl-2-associated X protein Bax (#5023), cytochrome c (#11940), caspase-3 (#9662), caspase-7 (#9492), caspase-8 (#4790), mitochondrial serine protease HtrA2/Omi (#9745), Poly (ADP-ribose) Polymerase PARP (#9542), p53 upregulated modulator of apoptosis PUMA (#12450), second mitochondria-derived activator of caspase (Smak/Diablo) (#2954), Fas-associated death domain FADD (#2784), Fas cell surface death receptor Fas (#4233), receptor-interacting protein kinase RIP (#3493), tumor necrosis factor receptor-1 TNFR1 (#3736), tumor necrosis factor receptor type 1-associated death domain TRADD (#3684), serine/threonine kinase Akt1 (#4691), NF- κ B p65 (D14E12) XP[®] (#8242), p38 MAPK (D13E1) XP[®] (#8690), p44/42 MAPK (#4695), tumor protein p53 (#2527), stress-activated protein kinase SAPK/JNK (#9252), and β -actin ACTB (#4970) as a reference protein (Cell Signaling Technology). Finally, the appropriate secondary antibody conjugated with horseradish peroxidase (#7074 or #7076, Cell Signaling Technology) was applied. Detection was executed by chemiluminescence, using ClarityTM Western ECL Substrate (Bio-Rad). Visualization of the detected protein was performed using the ChemiDocTM Imaging System (Bio-Rad). Densitometric assays were performed using ImageJ (author Wayne Rasband). Results are shown as a mean \pm SD normalized to the internal reference protein (β -actin). Untreated negative control (UC) was set as 100% expression level.

2.11. Statistical Analysis

All experiments were performed as at least three independent experiments and measured in triplicate. A Shapiro–Wilk’s test was applied to assess the normality of distribution. *T*-test was applied to compare unpaired means between two groups and $p \leq 0.05$ was regarded as significant. Based on obtained results for Muse[®] flow cytometer analysis, one-way analysis of variance (ANOVA) was carried out. Duncan post-hoc test was used for testing the differences and $p \leq 0.05$ was regarded as significant. Statistical analysis was performed using Statistica v.13.3 software (Stat-Soft, Tulsa, OK, USA).

3. Results and Discussion

Greater knowledge about prostate cancer has led to the opinion that this is a preventable disease and role of diet has gained considerable attention. Results of case-control studies has shown that the intake of cruciferous vegetables significantly decrease risk of prostate cancer [15]. Health-promoting, including antitumor properties, of individual compounds of red cabbage are well described [16,17]. However, this approach does not take into account how single components interact with one another, which involves inhibiting

or enhancing their antitumor activity. Vegetable bioactive compounds are particularly attractive because of human long-standing exposure to them, their relative lack of toxicity, and encouraging indications from epidemiological studies [18]. Natural chemopreventative agents should be highly effective, easy to administer, and inexpensive. Edible parts of cruciferous plants can be a natural source of phytochemicals with proven health properties. This includes young shoots or sprouts of red cabbage that do not require intensive development, new equipment, or expensive marketing [19].

3.1. Cytotoxicity and Proliferation

Studied juices subjected to *in vitro* gastrointestinal digestion and absorption showed a cytotoxic effect on examined cell lines (Table 1). Cytotoxicity levels for juice of young shoots of red cabbage were lower than those observed in the juice of mature cabbage (Table 1). The cytotoxicity results for treatment with both juices were the lowest for the PNT-2 normal prostate cell line (Table 1). In the current study, we observed that juices subjected to *in vitro* gastrointestinal digestion and absorption statistically significantly ($p \leq 0.05$) inhibited the proliferation of DU145 and LNCaP cell lines compared to the untreated control (Figure 1). Additionally, it showed that the juice of young shoots decreased the proliferation of study cell lines more effectively than the juice of mature cabbage (Figure 1). After 24 h of incubation of DU145 cancer cells with both juices, proliferation significantly decreased ($p \leq 0.05$, Figure 1). Proliferation was reduced by approximately 32% and 27% for treatment with the juice of young shoots and of mature vegetables, respectively (Figure 1). The results for LNCaP prostate cancer cell line were prominent and showed a high level of sensitivity to both juices. Statistically significant ($p \leq 0.05$) inhibition of proliferation by 44% and 37% was observed after 24-h incubation of cells with the digested and absorbed juice of young shoots and of the vegetable at full maturity, respectively (Figure 1). Interestingly, PNT-2 normal prostate cells did not show a reduction in proliferation after treatment with both juices (Figure 1). No changes in proliferation were observed for PNT-2 normal prostate cells. Consequently, it was decided not to study mechanisms of cell death. We obtained similar results in our previous studies, while testing the fresh juices of the tested vegetable juice of young shoots reduced the proliferation of both cell lines more effectively than the juice of mature cabbage [11]. Kestwal et al. [20] determined that aqueous extracts of cabbage sprouts had high anti-proliferative activity against HepG2 human hepatocarcinoma cells and CT26 mouse colorectal cancer cells. In our previous research, we showed that the fresh juice of young shoots of white-headed cabbage decreased the viability of MCF-7 breast cancer cells [21]. Roy et al. [22] revealed that juice of white cabbage showed the highest antiproliferative activity in HL-60 cells. Boivin et al. [23] observed that red cabbage was one of the most active cruciferous vegetables, with complete inhibition of the proliferation of most cancer cell lines (also PC3 prostate cancer cell line). Other research on the cytotoxicity effect of red cabbage extract showed a decrease in the viability of human A-549, HT29, and HCT116 cell lines [24].

Table 1. Cytotoxicity of studied juices.

DU145 Cytotoxicity [%]		LNCaP Cytotoxicity [%]		PNT-2 Cytotoxicity [%]	
ys d + a vs. UC ± SD	mv d + a vs. UC ± SD	ys d + a vs. UC ± SD	mv d + a vs. UC ± SD	ys d + a vs. UC ± SD	mv d + a vs. UC ± SD
6.44 ± 2.51	7.05 ± 2.88	7.27 ± 1.53	7.9 ± 1.95	4.09 ± 1.43	5.37 ± 1.74

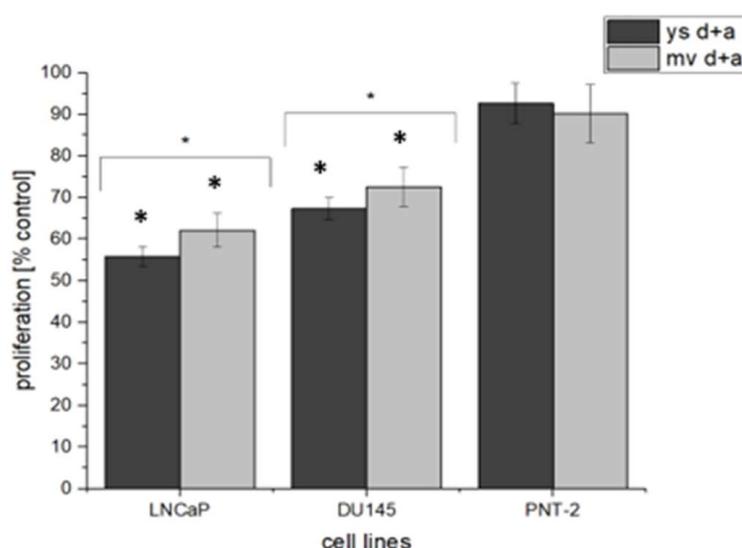


Figure 1. Effect of juice of young shoots of red cabbage (ys d + a) and of the mature vegetable (mv d + a) subjected to in vitro gastrointestinal digestion and absorption on DU145 and LNCaP prostate cancer cells and PNT-2 normal cancer cells proliferation in test with using Cell Proliferation ELISA, BrdU (Sigma-Aldrich). Values are expressed as means \pm SD for $n = 9$, standardized to an untreated control set as 100%. Statistical significance was based on t -test * $p \leq 0.05$.

A reduction of cell growth and an induction in cell death are two major means to inhibit tumor growth. Apoptosis is a gene-directed type of cell death and has become the major indicator through which chemopreventive and chemotherapeutic factors inhibit the growth of cancer cells. There is increasing evidence that apoptosis plays a major role as a defense against cancer, so understanding its mechanism has important implications in the prevention and treatment of many cancers [25]. Thus, we investigated whether studied juices may be involved in the induction of apoptosis, apart from cell proliferation inhibition.

3.2. Analysis of Apoptosis Using the Muse® Flow Cytometer

Flow cytometry with the Muse® Annexin V & Dead Cell Assay, Muse® MultiCaspase Assay Kit, and Muse® Bcl-2 Activation Dual Detection Assay Kit was used to investigate the mode of studied juices-induced cell death in DU145 and LNCaP prostate cell lines.

The Muse® Annexin V & Dead Cell Assay for quantitative analysis of live, early/late apoptotic, and dead cells was used. Annexin V is a calcium-dependent cytoplasmic protein with a high affinity for phosphatidylserine, inner cell membrane component. During apoptosis, annexin V can bind phosphatidylserine, which translocates to the outer surface of the cell membrane [26]. In this research, we also performed multi-caspase activity (caspase-1, -3, -4, -5, -6, -7, -8, and -9) to confirm the apoptosis mechanism. The Muse® MultiCaspase Assay Kit determines the count and percentage of cells with caspase activity, in combination with a dead cell dye. The detection assays revealed a significant ($p \leq 0.05$) decrease in live cells in the examined juices treatment in comparison to the medium control in DU145 and LNCaP prostate cancer cell lines (Tables 2–5; Supplementary Figures S1 and S2). Additionally, there was a significant ($p \leq 0.05$) increase in the total apoptosis rate and number of caspase positive cells compared to the untreated control in both cell lines (Tables 2–5; Supplementary Figures S1 and S2). In DU145 and LNCaP cell lines, after treatment of the young shoots' juice that was subjected digestion and absorption, the total apoptosis rate was $44.50 \pm 2.83\%$ and 55.37 ± 0.57 , respectively (Tables 2 and 4; Supplementary Figures S1 and S2). After treatment with juices, the rate of early apoptosis exceeded that of the late, however, for the young shoots' juice subjected to in vitro digestion and absorption in the LNCaP cell line, late apoptosis was predominant (Tables 2 and 4; Supplementary Figures S1 and S2). We found that the number of caspase positive cells was increased in DU145 and LNCaP cell lines and this activation depended on the juice used (Tables 3 and 5;

Supplementary Figures S1 and S2). It was observed that young shoots' juice subjected to in vitro gastrointestinal digestion and absorption increased the number of caspase positive cells more effectively in comparison to the digested and absorbed juice of the mature vegetable in both cell lines (Tables 3 and 5; Supplementary Figures S1 and S2). Similar results were noticed for the juice of young shoots not subjected to the aforementioned processes (Tables 3 and 5; Supplementary Figures S1 and S2). Similar to our study, it has been stated that bioaccessible fractions of broccoli, mustard, and radish microgreens show induction of early apoptosis in Caco-2 cells [27]. Similarly, extracts of fresh green cabbage, compared with the control group, induced cell apoptosis in A529 cells by increasing early apoptosis [28]. Hafidh et al. [29] showed that red cabbage extract inhibits HeLa and HepG2 cancer cell lines growth through the induction of apoptosis via caspase-dependent pathway. According to Singh et al. [30], glucosinolates induce caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells.

Table 2. Cell apoptosis results of DU145 prostate cancer cells treated with experimental juices. The apoptosis profiling and apoptotic cell counts were obtained using flow cytometry (Muse[®] Cell Analyzer) and Muse[®] Annexin V and Dead Cell Assay Kit.

	UC	STS	ys d + a	ys%	mv d + a	mv%
live	94.90 ^d ± 0.30	67.18 ^c ± 0.38	54.44 ^a ± 2.84	61.50 ^b ± 0.37	55.69 ^a ± 0.69	68.04 ^c ± 1.84
early apoptotic	4.00 ^a ± 0.25	30.10 ^d ± 0.70	25.17 ^c ± 3.30	32.54 ^d ± 0.47	30.63 ^d ± 4.93	20.24 ^b ± 1.77
late apoptotic	1.03 ^a ± 0.08	2.62 ^a ± 1.06	19.34 ^d ± 0.47	5.93 ^b ± 0.80	13.44 ^c ± 4.10	11.63 ^c ± 0.10
dead	0.08 ^a ± 0.03	0.11 ^a ± 0.02	1.07 ^c ± 0.00	0.04 ^a ± 0.04	0.24 ^b ± 0.14	0.10 ^a ± 0.03
total apoptotic	5.03 ^a ± 0.33	32.72 ^b ± 0.36	44.50 ^d ± 2.83	38.47 ^c ± 1.33	44.07 ^d ± 0.83	31.87 ^b ± 1.87

UC, untreated control. STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as mean ± SD ($n = 3$). Statistically significant differences (based on ANOVA and Duncan's a posteriori test) $p \leq 0.05$ within each row are indicated by letter code a–d.

Table 3. Cell apoptosis results of DU145 prostate cancer cells treated with experimental juices. Apoptosis was analyzed using flow cytometry (Muse[®] Cell Analyzer) and the Muse[®] MultiCaspase Assay Kit.

	UC	STS	ys d + a	ys%	mv d + a	mv%
live	93.55 ^f ± 0.50	33.05 ^a ± 0.25	37.33 ^b ± 0.22	77.38 ^d ± 1.88	39.28 ^c ± 0.68	79.73 ^e ± 0.27
caspase+	5.53 ^a ± 0.18	40.18 ^d ± 0.28	41.43 ^c ± 0.68	19.00 ^b ± 1.85	54.30 ^e ± 0.05	7.68 ^a ± 0.18
caspase+/dead	0.78 ^a ± 0.23	26.70 ^f ± 0.05	21.1 ^e ± 0.45	3.30 ^b ± 0.05	5.90 ^c ± 0.60	12.55 ^d ± 0.50
dead	0.15 ^b ± 0.05	0.08 ^{a,b} ± 0.03	0.15 ^e ± 0.0	0.33 ^c ± 0.07	0.53 ^d ± 0.03	0.05 ^a ± 0.05
total caspase	6.30 ^a ± 0.05	66.88 ^f ± 0.22	62.53 ^e ± 0.23	22.30 ^c ± 1.80	60.20 ^d ± 0.65	20.23 ^b ± 0.33

UC, untreated control. STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as mean ± SD ($n = 3$). Statistically significant differences (based on ANOVA and Duncan's a posteriori test) $p \leq 0.05$ within each row are indicated by letter code a–f.

Table 4. Cell apoptosis results of LNCaP prostate cancer cells treated with experimental juices. The apoptosis profiling and apoptotic cell counts were obtained using flow cytometry (Muse[®] Cell Analyzer) and the Muse[®] Annexin V and Dead Cell Assay Kit.

	UC	STS	ys d + a	ys%	mv d + a	mv%
live	94.58 ^e ± 0.27	64.57 ^d ± 1.64	42.4 ^a ± 1.00	55.34 ^c ± 1.67	51.25 ^b ± 2.85	62.93 ^d ± 0.60
early apoptotic	4.18 ^a ± 0.43	30.17 ^{c,d} ± 1.77	22.4 ^b ± 2.87	33.53 ^c ± 1.40	29.45 ^{c,d} ± 6.00	27.33 ^{b,d} ± 0.20
late apoptotic	0.88 ^a ± 0.08	5.03 ^{a,b} ± 0.23	32.97 ^d ± 2.30	10.96 ^b ± 0.23	19.05 ^c ± 8.65	9.6 ^b ± 0.87
dead	0.1 ^a ± 0.05	0.23 ^a ± 0.11	2.24 ^b ± 0.43	0.17 ^a ± 0.04	0.25 ^a ± 0.20	0.14 ^a ± 0.07
total apoptotic	5.33 ^a ± 0.23	35.20 ^b ± 1.54	55.37 ^e ± 0.57	44.5 ^c ± 1.63	48.5 ^d ± 2.65	36.94 ^b ± 0.66

UC, untreated control. STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as mean ± SD ($n = 3$). Statistically significant differences (based on ANOVA and Duncan's a posteriori test) $p \leq 0.05$ within each row are indicated by letter code a–e.

Table 5. Cell apoptosis results of LNCaP prostate cancer cells at treated with experimental juices. Apoptosis was analyzed using flow cytometry (Muse[®] Cell Analyzer) and the Muse[®] MultiCaspase Assay Kit.

	UC	STS	ys d + a	ys%	mv d + a	mv%
live	87.83 ^d ± 2.78	34.25 ^a ± 1.55	30.28 ^a ± 2.08	58.65 ^b ± 0.35	62.2 ^b ± 9.35	78.73 ^c ± 0.57
caspase+	8.68 ^a ± 0.77	38.50 ^d ± 14.95	22.13 ^{b,c} ± 1.73	15.55 ^{a,b,c} ± 0.15	25.2 ^c ± 3.75	12.05 ^{a,b} ± 0.05
caspase+/dead	3.28 ^a ± 1.98	26.83 ^b ± 16.68	47.55 ^c ± 0.35	25.80 ^b ± 0.20	12.4 ^a ± 5.65	9.08 ^a ± 0.48
dead	0.23 ^c ± 0.03	0.43 ^d ± 0.18	0.05 ^{a,b} ± 0.0	0.0 ^a ± 0.0	0.2 ^{b,c} ± 0.05	0.1 ^{a,b,c} ± 0.1
total caspase	11.95 ^a ± 2.75	63.60 ^d ± 2.01	69.68 ^d ± 2.08	41.35 ^c ± 0.35	37.6 ^c ± 9.4	21.18 ^b ± 0.48

UC, untreated control. STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as mean ± SD ($n = 3$). Statistically significant differences (based on ANOVA and Duncan's a posteriori test) $p \leq 0.05$ within each row are indicated by letter code a–d.

Mitochondrial dysfunction is associated with the intrinsic pathway of apoptosis, which is triggered by several non-receptor mediated stimuli causing changes in the internal mitochondrial membrane. It results in the opening of a permeability transition pore complex, loss of mitochondrial potential, and release of pro-apoptotic molecules (e.g., cytochrome c, Smac/Diablo, and HtrA2/Omi) [31]. The Bcl-2 family proteins are major regulators of this pathway of apoptosis. The Muse[®] Bcl-2 Activation Dual Detection Assay Kit determines the content of activated and non-activated Bcl-2 in cells, a protein on which the survival of cancer cells depends. All examined juices induced in DU145 and LNCaP cells the inactivation of the Bcl-2 protein (Figure 2). A higher percentage of cells with the inactivation of the above-mentioned protein, in comparison to untreated control, was observed. Highly significant ($p \leq 0.05$) changes in the phosphorylation of the studied protein in both cell lines were shown after incubation with the juice of young shoots subjected to in vitro gastrointestinal digestion and absorption; a decrease in Bcl-2 phosphorylation to about 83% compared to the control was shown (Figure 2). It was also observed that the juice of young shoots inactivated the Bcl-2 protein more effectively than that of the mature cabbage's juice (Figure 2).

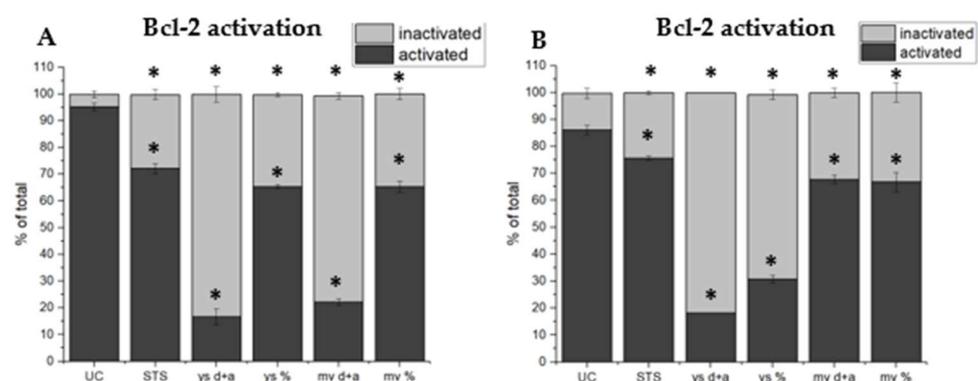


Figure 2. Cell apoptosis results of DU145 (A) and LNCaP (B) prostate cancer cells treated with experimental juices. Bcl-2 activation were obtained using flow cytometry (Muse[®] Cell Analyzer) and the Muse[®] Bcl-2 Activation Dual Detection Assay Kit. UC, untreated control. STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as mean ± SD ($n = 3$). Statistical significance was based on ANOVA and Duncan's a posteriori test. * $p \leq 0.05$ compared to untreated control.

3.3. mRNA Expression of Genes and Western Blot Analysis of Protein Levels Associated with Proliferation and Apoptosis

Anti-apoptotic Bcl-2 family proteins mainly prevent the release of apoptogenic molecules from mitochondria to the cytosol by forming heterodimer with proapoptotic proteins [31].

The pro-apoptotic proteins, e.g., Bax, Bid, and PUMA, bind to the outer membrane of the mitochondria to signal the release of the internal proteins. In normal cells, the Bax protein is an inactive form in the cytosol and in response to certain apoptotic, can be induced to change conformation and translocate into the mitochondria [32]. A transcription factor, p53 plays, a relevant role in the regulation of the expression of Bcl-2 family proteins [33]. The *TP53* gene controls the transcription of many genes and regulates processes related to DNA repair, cell-cycle arrest, or apoptosis [34]. Our results for juice-treated cancer cells showed significant ($p \leq 0.05$) up-regulation of the *TP53* gene and the p53 protein in both examined cell lines (Table 6, Figures 3 and 4; Supplementary Figures S3 and S4). In addition, we determined that *BBC3* (the p53 upregulated modulator of apoptosis, *PUMA*), *BAD* (Bcl-2 associated agonist of cell death), and *DIABLO* mRNA level in the DU145 cell line was significantly ($p \leq 0.05$) increased after exposure to juices, especially to the juice of young shoots, both fresh and subjected to in vitro digestion and absorption (Table 6). The *BBC3* gene is the essential mediator of p53 dependent apoptosis. The BAD protein induced cell death, successfully competing for the binding to Bcl-xL, Bcl-2, and Bcl-w, affected the level of heterodimerization of these proteins with Bax [35].

Table 6. mRNA expression of genes.

Gene Symbol	DU145				LNCaP			
	ys d + a vs. UC	ys% vs. UC	mv d + a vs. UC	mv% vs. UC	ys d + a vs. UC	ys% vs. UC	mv d + a vs. UC	mv% vs. UC
<i>AIFM1</i>	10.888 **	5.813 **	7.402 **	4.843 **	5.971 **	2.399 **	1.474	3.645 **
<i>AKT1</i>	0.105 **	0.860	0.117 **	1.874	0.221 **	0.281 **	0.209 **	1.006
<i>Apaf-1</i>	6.649 **	1.198	4.854 **	0.952	1.545 *	Ns	3.162 **	Ns
<i>BAD</i>	2.041 *	2.397 *	1.676 *	2.618 **	1.300	1.891	1.600	1.055
<i>BBC3</i>	4.987 **	2.346 **	1.641 *	1.422 *	Ns	Ns	Ns	Ns
<i>CASP-3</i>	10.997 **	1.550	5.606 **	1.429 *	2.232	1.844	4.721 **	1.120
<i>CASP-7</i>	8.893 **	2.511 **	2.498 **	0.754	3.305 **	2.520 *	2.696 **	1.194
<i>CASP-8</i>	5.748 **	0.462 **	1.503 **	0.583	Ns	Ns	Ns	Ns
<i>DIABLO</i>	4.901 **	2.481 **	4.113 **	6.920 **	Ns	Ns	Ns	Ns
<i>FADD</i>	2.242 **	1.500	2.152 *	1.483	0.998	0.773	0.621 **	0.619 **
<i>FAS</i>	6.617 **	1.124	4.812 **	3.216 *	0.766	0.058	0.837	2.938 *
<i>TP53</i>	4.821 **	1.219 **	1.076	2.756 **	1.802 **	1.524 **	1.809 **	1.502 **

mRNA expression of selected genes in DU145 and LNCaP prostate cancer cells after treatment with: The juice of young shoots of red cabbage (ys d + a) and of the mature vegetable (mv d + a) subjected to in vitro gastrointestinal digestion and absorption; fresh, 5% juice of young shoots (ys%) and fresh, 5% juice of the mature vegetable (mv%). UC, untreated control. Ns, no signal. Statistical significance was based on *t*-test: ** statistical significance of treatment $p \leq 0.05$; * statistical tendency of treatment $p \leq 0.1$.

Using Western blot analysis, we found a significant increase ($p \leq 0.05$) in the pro-apoptotic Bcl-2 family members-Bax and PUMA proteins expression in DU145 and LNCaP prostate cancer cells treated with the experimental juices (Figures 3 and 4; Supplementary Figures S3 and S4). A higher expression of these proteins was observed in the DU145 cell line treated with the juice of young shoots subjected to in vitro gastrointestinal digestion and absorption in comparison to that treated with juice of the mature cabbage subjected to the same processes (Figures 3 and 4; Supplementary Figures S3 and S4). The current results revealed that the juices up-regulated ($p \leq 0.05$) the expression of cytochrome c, Smac/Diablo, and HtrA2/Omi proteins (Figures 3 and 4; Supplementary Figures S3 and S4). However, the LNCaP cell line did not express the Smac/Diablo protein. It has been stated that the release from the mitochondria of the mentioned above proteins could be controlled by Bax and the translocation of Bax can alter the outer mitochondrial membrane's permeability, and then activate the intrinsic apoptosis pathway. Another pro-apoptotic protein released from the mitochondria during apoptosis is AIF (apoptosis-inducing factor), which translocates to the nucleus and causes DNA fragmentation and chromatin condensation [36]. Our results also showed significant ($p \leq 0.05$) increase in the *AIFM1* protein coding gene mRNA expression in both cell lines (Table 6). Following the release, cytochrome c forms a complex in the cytoplasm with ATP and apoptotic protease-activating

factor-1 (Apaf-1). This complex will activate caspase-9 and the apoptosome is formed, which, in turn, activates caspase-3, the effector protein that initiates degradation. In the current study, we determined the up-regulation ($p \leq 0.05$) of the *Apaf-1* gene, in cell lines especially after treatment with juices subjected to in vitro gastrointestinal digestion and absorption (Table 6). *Apaf-1* encodes a cytoplasmic protein that initiates apoptosis, and accordingly, we observed the up-regulation ($p \leq 0.05$) of the Apaf-1 protein expression in the DU145 cell line (after treatment with juices subjected to in vitro gastrointestinal digestion and absorption increased) and in the LNCaP cell line after treatment with juices, excluding fresh mature cabbage juice (Figures 3 and 4; Supplementary Figures S3 and S4).

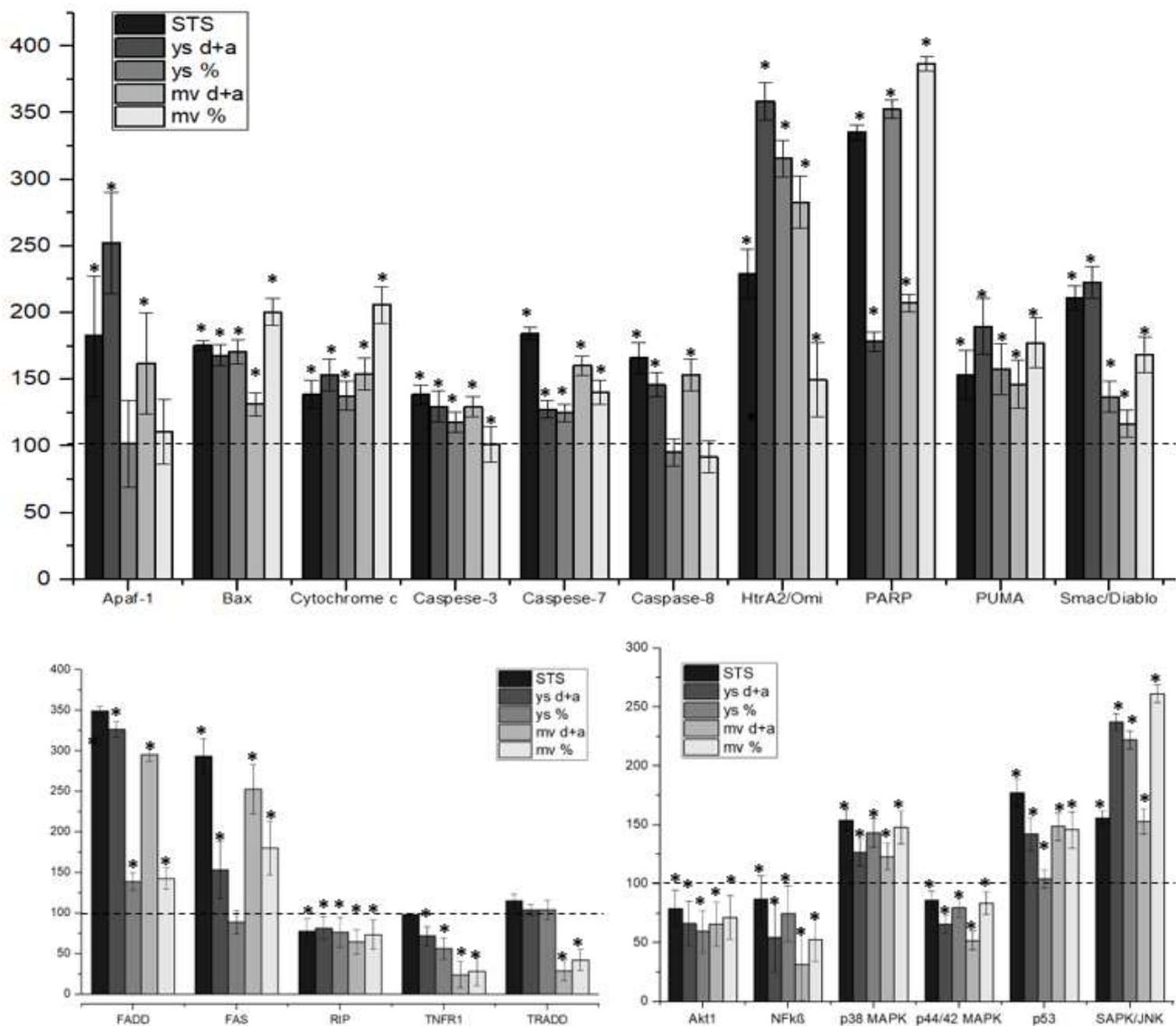


Figure 3. Expression of proteins involved in proliferation and apoptosis signaling in DU145 prostate cancer cells. DU145 prostate cancer cells were treated for 24 h with: STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as a mean \pm SD normalized to the internal reference protein (β -actin). Untreated negative control (UC) was set as 100% expression level. Statistical significance was based on *t*-test * $p \leq 0.05$ vs. UC.

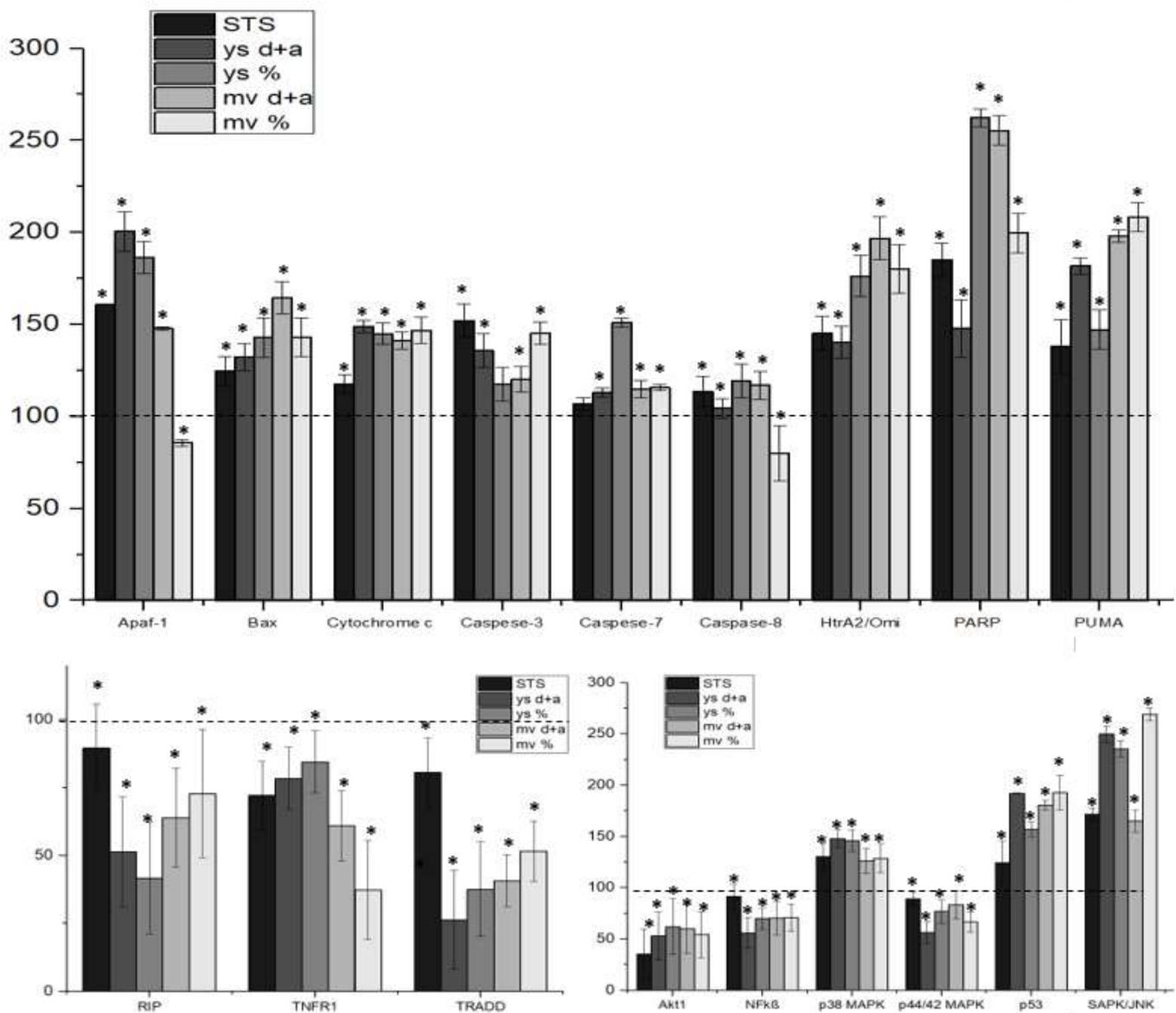


Figure 4. Expression of proteins involved in proliferation and apoptosis signaling in LNCaP prostate cancer cells. LNCaP prostate cancer cells were treated for 24 h with: STS, staurosporine. ys d + a, juice of young shoots subjected to in vitro gastrointestinal digestion and absorption. ys%, fresh, 5% juice of young shoots. mv d + a, juice of the mature vegetable subjected to in vitro gastrointestinal digestion and absorption. mv%, fresh, 5% juice of the mature vegetable. Results are expressed as a mean \pm SD normalized to the internal reference protein (β -actin). Untreated negative control (UC) was set as 100% expression level. Statistical significance was based on t-test * $p \leq 0.05$ vs. UC.

The extrinsic or death receptor pathway involves the binding of signal ligands to cell surface death receptors, members of the tumor necrosis factor receptor gene (*TNFR*) superfamily, and inducing apoptosis [37]. Upon binding, the ligand to the Fas death receptor, the cytoplasmic adaptor protein FADD, exhibiting corresponding death domains, is recruited. Procaspase-8 then is recruited, forming a death-inducing signaling complex (DISC). Active caspase is then released from the DISC complex into the cytosol, and directly activates caspase-3 and -7 (effector proteins) to start the caspase cascade and initiate degradation of the cell. Active caspases can also activate the Bid protein and represent a “cross-talk” of the two main pathways, amplifying the apoptotic signaling from death receptors [37]. The current results revealed that juices up-regulated the expression of gene and protein involved in the extrinsic or death receptor pathway of apoptosis (Table 6; Figures 3 and 4; Supplementary Figures S3 and S4). In the DU145 cell line treated with

juices subjected to in vitro gastrointestinal digestion and absorption, an up-regulation ($p \leq 0.05$) expression of *FAS* and *FADD* genes was observed. For the LNCaP cell line, only after treatment with fresh juice of the mature vegetable was an up-regulation ($p \leq 0.05$) expression of *FAS* gene observed (Table 6). Furthermore, we found that the level of the FAS receptor and adaptor protein FADD expression in DU145 significantly ($p \leq 0.05$) increased (exception was expression of FAS after treatment of cells with fresh juice of young shoots) (Figure 3; Supplementary Figure S3). The LNCaP cell line did not express FAS and FADD proteins.

In the following binding of the TNF α to TNFR1 receptor and trimerisation, the adaptor molecule TRADD is recruited, which has the ability to recruit secondary adaptors such as RIP or TRAF2 (TNF receptor-associated factor 2). These, respectively, activate the pathways related with NF- κ B and JNK/AP-1 and can stop the apoptotic signal and provide cell survival [37]. Activation of NF- κ B by extracellular stimuli (e.g., TNF cytokine) leads to rapid phosphorylation, ubiquitination, and proteolytic degradation of its inhibitor protein (I κ B), thereby resulting in the nuclear translocation of NF- κ B and initiation of the transcription of a large number of, also anti-apoptotic, genes promoting survival [38]. In this study, juices significantly ($p \leq 0.05$) decreased the expression of the TNFR1 receptor, adaptor protein TRADD, and secondary adaptor protein RIP in both cell lines. Fresh juice of young shoots as well as being subjected to in vitro gastrointestinal digestion and absorption, had no effect ($p \geq 0.05$) on the TRADD level in the DU145 cell line (Figure 3; Supplementary Figure S3). Simultaneously, it was noted that the studied juices mediated ($p \leq 0.05$) down-regulation of the NF- κ B level in both prostate cancer cell lines (Figures 3 and 4; Supplementary Figures S3 and S4). Due to the fact that the mentioned proteins are involved in the activation of the nuclear factor- κ B and inhibition of NF- κ B activation is believed to suppress tumorigenesis and the progression of tumors, it can be suggested that the inhibition of prostate cancer cells proliferation and activation of programmed cell death, in this research, is related to this mechanism.

Caspases are a family of protease enzymes that are formed constitutively in the cells and are present as inactive proenzymes. These molecules are activated in the early stages of apoptosis in a self-amplifying cascade [31]. As mentioned above, two major pathways of caspase cascade activation have been differentiated (activation of caspase-8 in death receptor-induced apoptosis and of caspase-9 in mitochondrial pathway). Activation of initiator caspases leads to the proteolytic activation of effector caspases-3, -6, and -7. These effector caspases are responsible for the cleaving of one of the earliest nuclear enzymes- PARP [39]. The up-regulation of caspase-3, -7, and -8 genes in the treated DU145 and LNCaP cells with juices (especially in DU145 cell line after incubation with juice of young shoots subjected to in vitro gastrointestinal digestion and absorption) was observed (Table 6). Finally, we confirmed the up-regulation ($p \leq 0.05$) expression of pro-apoptotic proteins: Caspase-3, -7, and -8 under juices treatment. However, the fresh juices had no measurable effect ($p \geq 0.05$) on caspase-8 level in the DU145 cell line (Figure 3, Supplementary Figure S3). In case of the LNCaP cell line, the fresh juice of the mature vegetable significantly decreased ($p \leq 0.05$) the expression of the caspase-8 level (Figure 4, Supplementary Figure S4). We also observed an increased level of PARP in both cell lines (Figures 3 and 4; Supplementary Figures S3 and S4).

In the current work, we also assessed the effect of juices on selected proteins involved in the regulation of proliferation, cellular stress response, and their links to apoptosis induction. Our results showed that the juices subjected to in vitro gastrointestinal digestion and absorption led to a down-regulation ($p \leq 0.05$) of the *AKT1* gene in DU145 and LNCaP cell lines (Table 6). This gene encodes the Akt1 protein, one of the members of the human Akt serine-threonine protein kinase family. One of the effector mechanisms in the cell, which plays a crucial role in signaling pathways important to cell survival and proliferation is the pathway triggered by the phosphatidylinositol 3-kinase (PI3K) and the protein kinase Akt. In simple terms, PI3K activates kinase Akt, which is involved in the activation of proteins that promote cell survival, e.g., the nuclear factor κ B. The main consequence of

its activation is the increased proliferation and tumor transformation and inhibition of apoptosis through its ability to phosphorylate and to inactivate several proteins, including Bad and caspase-9 [40]. In our study, we also noted that treatment with juices significantly ($p \leq 0.05$) decreased the level of Akt1 and p44/42 MAPK protein in prostate cancer cell lines (Figures 3 and 4; Supplementary Figures S3 and S4). p38 MAPK and SAPK/JNK MAPKs are activated by a wide range of cellular stressors. SAPK/JNK can promote the activation of both, external and mitochondrial apoptosis pathways. It has been suggested that these mechanisms can act independently or co-operate with one another to induce cell death [41]. Our results indicated a significantly ($p \leq 0.05$) decreased level of p38 MAPK and SAPK/JNK MAP kinases in both DU145-7 and LNCaP cell lines after treatment with juices. A higher expression of these proteins was observed after treatment with the juice of young shoots subjected to in vitro gastrointestinal digestion and absorption in comparison to that after treatment with the juice of red cabbage subjected to the same processes (Figures 3 and 4; Supplementary Figures S3 and S4).

Although a great number of research dealing with the anticarcinogenic activity of single components have been conducted, only a few studies were performed using whole Brassica vegetables, including red cabbage and in particular their young shoots. Research by Hafidh et al. [29] on red cabbage extract may support our findings regarding the activation of different apoptotic pathways in cancer cells. They reported that red cabbage extracts up-regulated the expression of caspase-7, -8, -9, and the *Bax* gene, and also down-regulated the expression of the *Bcl-2* gene in both HeLa and HepG2 cells. Red cabbage extract was also found to induce apoptosis in the human cancer cells via caspase-dependent pathway [42]. Nam and Kang [43] investigated that the red cabbage extract induced cell death in MDA-MB-231 human breast cancer cells, decreased the expression of *Bcl-2* gene, and increased the expression of *Bax* and caspase-3 genes.

In our previous publication, we showed that the young shoots of red cabbage, from which the juice was obtained for the study described in the current manuscript, contain many glucosinolates, polyphenols, vitamin C, and carotenoids [10,11]. Glucosinolates may break down in plant material during processing through endogenous enzyme myrosinase action, or within the gastrointestinal tract through the action of commensal microflora. A proportion of these breakdown products enter the epithelial cells of the gastrointestinal mucosa, undergo metabolism, enter to the circulation, and then undergo further metabolism in the liver [44]. Cruciferous sprouts, including young shoots of red cabbage, could be a good dietary source of aliphatic glucosinolates, glucoraphanin being one of the most abundant, and its derivative-sulforaphane, which was reported to induce apoptosis in the human colon cancer cell line HT29 in the intrinsic pathway [45]. Moreover, sulforaphane up-regulated the *Bax* protein in LNCaP cells, down-regulated the expression of *Bcl-2* in DU145 cells, and inhibited NF- κ B transcriptional activity in PC-3 [16,46,47]. Another GLS present in the studied vegetable is glucoiberin (precursor of the isothiocyanate iberin), which has beneficial effects on oxidative stress and cancer prevention [7,10]. A high concentration of total indole GLS, represented by glucobrassicins, neoglucobrassicin, and 4-methoxyglucobrassicin, was observed in young shoots [10,48]. Sarkar and Li [49] demonstrated that indole-3-carbinol (I3C; derived from the breakdown of glucobrassicin) regulated many genes involving in cell cycle regulation, proliferation, and other cellular processes, suggesting the pleiotropic effects of this compound on the prostate cancer cell line. Chinni et al. [50] and Nachshon-Kedmi et al. [51] reported that I3C was an effective agent in the inhibition of cell growth and induction of programmed cell death of PC-3 and of DU145 and LNCaP prostate cancer cells, respectively. The breakdown product of the 4-methoxyglucobrassicin (4-methoxyindole-3-carbinol) might play a role as a chemopreventive agent, inhibiting cell growth and causing cell death of human cancer cells in vitro [52].

Phenolic extracts and single polyphenols from different plant foods have been studied in a number of cancer cell lines representing different developmental stages of cancer. Polyphenols exert their biological actions by different but complementary mechanisms. A

study demonstrated the anti-proliferating effects of polyphenols, alone or in combination, on cancer cells lines, like breast (T47D, MCF7, MDA-MB-231) and prostate (DU145, PC3, LNCaP), in a time and dose-response manner [53]. These compounds can influence cellular and molecular mechanisms related to carcinogenesis, such as the inhibition of factor NF- κ B, induction of cell cycle arrest or apoptosis through initiation of caspase-3-dependant pathway or due to activation of p53, affecting cell proliferation and differentiation or chemical metabolism [54]. Phenolic acids and flavonoids, including anthocyanins, were found in young shoots, in mature red cabbage and in juices obtained from them (also in juices used in this study) [11]. Phenolic acids have been shown to prevent angiogenesis and inhibit the initiation and progression of cancers. Flavonoids possess antioxidative, anti-inflammatory, and also anticancer properties including the suppression of angiogenesis, induction of apoptosis and down-regulation of hormone receptor expression [55]. The synergistic effects of flavonoids on the inhibition of LNCaP cells proliferation have also been studied [56]. The data from cell viability assay showed that anthocyanins exhibited a potent anti-proliferation effect on AGS, PC3, C4-2, and LNCaP cell lines and this process was related to the cell cycle arrest, modulation of NF- κ B signaling, or activation of apoptosis [57, 58]. The occurrence of apoptosis induced by anthocyanins was confirmed by morphological and biochemical features, including apoptotic bodies formation, down-regulating the Bcl-2 protein, and up-regulating levels of Bax protein, caspase-3 activation, poly(ADP-ribose) polymerase proteolysis, modulating the expression of FAS and increasing the p38 kinase expression, and inhibiting ERK activity [59,60].

4. Conclusions

In the current study, we showed that the juice of young shoots and of the mature red cabbage subjected to *in vitro* gastrointestinal digestion and absorption and not being subjected to these processes inhibited the proliferation of DU145 and LNCaP prostate cell lines. Moreover, we clearly demonstrated that the juices induced cell death through different apoptotic pathways. It is perhaps the first time that the mechanisms of cell death induced by the juices subjected to *in vitro* gastrointestinal digestion and absorption have been reported. The effect of juices on both DU145 and LNCaP cells was comparable but with minor differences. We provide evidence that the induction of apoptosis occurring in both cell lines by juices is exerted through the mitochondrial pathway. In addition, we observed the up-regulation of genes and proteins involved in the extrinsic or death receptor pathway of apoptosis in the DU145 cell line. Inhibition of NF- κ B and Akt activity may result in the inhibition of survival signals and also induce apoptotic signals in both cell lines. Additionally, the regulation of activity of stress-activated protein kinases may suggest their cooperation with the mentioned mechanisms to induce cell death. The possible reason behind these findings might be related to the fact that the juice was composed of different components that have pleiotropic anticarcinogenic activities and, thus, might trigger different pathways of apoptosis. Accordingly, using juice (as a source of all components) shows the advantage of triggering a few pathways of apoptosis simultaneously, which leads to the induction of apoptosis. The induction of multi-pathway apoptosis usually leads to effective anticancer activity.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app112311507/s1>, Table S1. Nucleotide sequences of primers, Figure S1. Cell apoptosis results of DU145 prostate cancer cells treated with experimental juices, Figure S2. Cell apoptosis results of LNCaP prostate cancer cells treated with experimental juices. Figure S3. Expression of proteins involved in proliferation and apoptosis signaling in DU145 prostate cancer cells, Figure S4. Expression of proteins involved in proliferation and apoptosis signaling in LNCaP prostate cancer cells.

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