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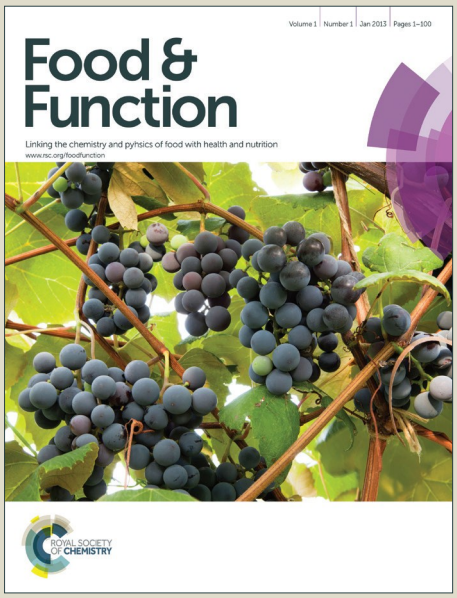

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PAPER

Betacyanins enhance vitexin-2-O-xyloside mediated inhibition of proliferation of T24 bladder cancer cells

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E. S. Scarpa,[a,†] M. Emanuelli,[b,c,†] A. Frati,[a] V. Pozzi,[b,c] E. Antonini,[a] G. Diamantini,[a] G. Di Ruscio,[b] D. Sartini,[b] T. Armeni,[b] F. Palma[a] and P. Ninfali[a,*]

Betacyanins (BC) were purified from beetroot (*Beta vulgaris* var. *rubra* L.) and tested, alone or in combination with vitexin-2-O-xyloside (XVX) from *Beta vulgaris* var. *cicla* L., for their ability to reduce the proliferation rate in T24 bladder cancer cells. Combination of BC and XVX exhibited a synergistic effect concerning the inhibition of proliferation in T24 cancer cells at 24 and 48 h but not after 72 h of incubation. The induction of apoptosis was evidenced by means of Fluorescence Activated Cell Sorting (FACS) analysis, as well as through the increase in caspase 3 and 8 activities. Using RTqPCR experiments, it was shown that the combination of XVX+BC was able to enhance the expression levels of pro-apoptotic BAX and downregulate anti-apoptotic BIRC5 (Survivin), as well as pro-survival CTNNB1 (β-Catenin). The most evident effect of BC was the increase of the activity of caspase 8, leading to induction of extrinsic apoptosis. Moreover, XVX, BC and their combination showed no cytotoxic effect in normal human skin NCTC 2544 keratinocytes. These results demonstrated the efficacy and the mechanisms of action of BC and XVX, extracted from edible plants, and suggest that a diet or a nutrition supplement, enriched with these bioactive molecules, could be used in prevention of human bladder cancer.

Introduction

The betalains are natural dyes present in the beetroot, *Beta vulgaris* var. *rubra* L. (*BVr*), which is utilized as a part of the regular diet in many countries [1]. Betalains are subdivided into betaxanthins (BX) and betacyanins (BC), both derived from betalamic acid [2]. In the beetroot, the BC are mainly represented by betanin and isobetanin, whereas in the BX group, the most important compounds are vulgaxanthin I and II [3]. The beetroot juice has been utilized in traditional medicine to cure a variety of diseases, with the therapeutic indication of hemostatic, renal protective and antitumor agent [4].

In the last decade, the betalains addressed a great interest, as the red dye E162, made with the beetroot extract, is one of the few red dyes admitted by the EFSA in food manufacturing, such as yogurt, ice creams, sauces and soups [5]. From the red dye E162 it is possible to purify a mixture of betanin and isobetanin, but complex mixtures of betacyanins and/or betaxanthins are purified from the beetroot. Several purification methods from the beetroot or other vegetables have been already published [1,2,6,7]. The strategy to develop purification methods is justified by the fact that the dye serves as a food quality indicator and researchers often investigated the degradation of betalains during transformation or cooking processes of beetroot and its derived products [7,8]. A betalain purification method could be useful to study the chemical and physical conditions that stabilize the dye [9], as well as to correlate the structure of the compounds with their biological activities [10].

A huge amount of research has been done on the biological activity of the beetroot juice and many studies confirmed the traditionally expected health protective effects [11,12].

For instance, the whole beetroot ethanolic extract was shown to attenuate renal dysfunction and structural damage through reduction of oxidative stress and inflammation in the kidney [13]. The anti-inflammatory activity was attributed to the ability of betalains to inhibit the NF-kB DNA binding [14] as well as to suppress the cyclooxygenase-2 expression [15].

Detailed investigations were also carried out to describe the anti-proliferative mechanisms of betanin and isobetanin. This topic was thoroughly investigated in human chronic myeloid leukemia p53 wt K562 cells, resulting in apoptosis induction by means of the intrinsic pathway [6]. Interestingly, Esatbeyoglu and colleagues [11] demonstrated that betanin exhibits a gene regulatory activity partly via Nuclear factor (erythroid-derived 2)-like-2 (Nrf2) dependent signaling pathways, inducing the expression of phase 2 enzymes and antioxidant defense mechanisms. Recently, it has also been demonstrated that betacyanins can induce both intrinsic and extrinsic apoptosis in MCF-7 breast cancer cells [16].

For several years, we have been investigating the anticancer activity of vitexin-2-O-xyloside (XVX), a C-glycosyl apigenin, which was purified by us from *Beta vulgaris* var. *cicla* L. (BVc) leaves and seeds [17,4]. XVX is considered an efficient

[a] Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy. E-mail: paolino.ninfali@uniurb.it; Fax: +39-0722-305324; Tel: +39-0722-305288
[b] Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy.
[c] New York-Marche Structural Biology Center (NY-MaSBiC), Polytechnic University of Marche, Ancona, Italy.
† Both authors contributed equally to this work.

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bioactive molecule, together with the companion molecules, vitexin and vitexin-2-O-rhamnoside [4]. In particular, studies have shown the antioxidant, anti-inflammatory, anti-viral and hypoglycemic activities of XVX, together with its anticancer effect [4]. XVX is able to induce apoptosis in RKO colon cancer cell lines [18] and in CaCo-2 and LoVo cancer cell lines [19].

On the basis of this groundwork, it would be possible that a combination of betalains (BX or BC) and XVX represents an efficient chemopreventive mixture, both through the diet and/or dietary supplements. Therefore, we decided to test the combination of betalains and XVX in order to evaluate the ability of the phytochemicals to inhibit T24 urinary bladder cancer cell proliferation [20]. T24 cancer cells are resistant to several anticancer drugs [21], due to their over expression of the Multi Drug Resistance system [22] and mutated TP53 gene, leading to the production of an inactive p53 protein, that cannot exert its anticancer action [23,24].

In this study, a group of betacyanins and betaxanthins were purified from BVr and tested for their antiproliferative activity, alone or in combination with XVX in T24 urinary bladder cancer cells. The aim of the study was to demonstrate the efficacy of our phytochemicals in the prevention and treatment of urinary bladder cancer and to describe the cellular and molecular mechanisms underlying the action of the combined mixture.

Materials and methods

Chemicals

Ethanol and ethyl acetate (analytical grade) were purchased from VWR International Inc. (West Chester, PA, USA). DMEM culture medium, foetal bovine serum (FBS), antibiotics, trypsin, water (LC-MS grade), acetonitrile (LC-MS grade) and formic acid (LC-MS grade) were purchased from Sigma-Aldrich Inc. (St.Louis, MO, USA).

Plant material

Seeds of *Beta Vulgaris cicla, cv. Bietola verde da taglio* (BVc) were obtained from Suba & Unico s.r.l. (Longiano, FC, Italy). *Beta Vulgaris rubra, cv. Detroit* (BVr) roots were cultivated by us in Castel Cavallino (Urbino, PU) and harvested at standard commercial maturity.

Purification of betalains

The beet roots were grated and extracted (1:10 w/v) with 70% ethanol in 10 mM Na-acetate buffer pH 5.0 for 2 hours under stirring at +6°C. The extract was filtered and concentrated by rotavap until it reached 10% of the initial volume. The remaining aqueous extract containing sugars, oxalic acids, phenolic acids and betalains was lyophilized and stored at -80°C. Betalains subfamilies were isolated by means of DEAE Sepharose fast flow (DEAE FF) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) chromatography, performed by means of the LC-Chromatograph AKTA Purifier 10. The lyophilized extract (5 g), suspended in 10 mM Na-phosphate buffer pH 7.5, was loaded onto the column (5.0 x 30 cm) and eluted first with a NaCl gradient from 0 to 0.08M, then from 0.08 M to 0.25 M NaCl in 10 mM Na-phosphate buffer pH 7.5. Betalains were assayed by reading the absorbance at 534 nm and 475 nm, using an extinction coefficient at 1% i.e $E^{1cm}_{1\%}$=1120 at 540 nm for BC and $E^{1cm}_{1\%}$=750 at 480 nm for BX [25]. The chromatographic profile of the DEAE FF column, with the applied gradient, is shown in Fig. 1. Three fractions were obtained: R1, R2 and R3. After lyophilization, the fractions were dissolved in water and loaded onto a Sephadex G10 column chromatography (1.5 x 90 cm) and eluted with distilled water [7]. The desalted fractions were lyophilized and used for HPLC analysis.

Analysis by HPLC-DAD-MS

R1, R2 and R3 samples were dissolved into the mobile phase and analyzed using a Water instrument equipped with Alliance HT 2795 High Performance Liquid Chromatography (HPLC), 2996 Diode Array Detector (DAD) and Micromass LC/MS ZQ 2000 detector, following the known procedure [26]. Data were processed using MassLynx 4.1 (Waters, Milford, USA). To identify betalains in the HPLC chromatograms, retention time, UV spectra, MS ESI(+) spectra were compared with data in literature [7].

Purification of XVX

Purification of XVX was performed from BVc seed extract 18 by using a Diaion HP 20 column (5 x 95 cm), followed by a Davisil C18 (2.5 x 14 cm) column, made by means of an LC-Chromatograph AKTA Purifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The elution of XVX was monitored at 336 nm and a peak fraction containing 95% XVX [18] was used for all the experiments.

Cell cultures

T24 human urinary bladder cancer cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 µg/ml gentamicin. NCTC 2544 keratinocytes cell line from normal human skin was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Both cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Sulforhodamine B (SRB) assay and synergy evaluation

The SRB assay is based on the ability of SRB dye to bind basic protein amino acid residues. The amount of dye incorporated by the cells indicates the cell number. Cells ($5x10^3$/well) were plated in 96-well plates and treated with XVX, BX, BC, the combination BX+BC and the combination XVX+BC for 24, 48 and 72 hours. At the end of phytochemical treatment, the cell culture medium was removed, and the cells were treated as

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previously described [19]. The results were expressed as a percentage of cells viability, compared to control. To evaluate the synergism between the phytochemicals, the measured cell viability (V%) was converted into % of growth inhibitory effect (GIE) by the calculation: % GIE = (100-V%), and this value was taken as an indication of the cytotoxic effect. The experimentally "Measured Cytotoxicity" in each mixture was divided by the "Expected Cytotoxicity", which is the mathematical sum of the cytotoxicity derived from the individual phytochemicals. If measured values were significantly higher than expected values, i.e. Measured/Expected Ratio > 1.0 ($p<0.05$), a synergistic effect was considered to have occurred; a Measured/Expected Ratio = 1.0 ($p<0.05$) indicated an additive interaction; a Measured/Expected ratio < 1.0 ($p<0.05$) indicated an antagonistic effect.

Apoptosis assessment by FACS analysis

Apoptosis was analyzed by flow cytometry, using the Annexin V-FITC Apoptosis Detection kit (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions [27]. Samples were analyzed using the Beckman Coulter EPICS XL flow cytometer. For each sample 10000 events were acquired. Annexin V-FITC is detected as a green fluorescent signal and propidium iodide is detected as a red fluorescent signal. The data acquired were quantified as percentages and analyzed by the FCS Express Program (De Novo Software, CA, USA).

RNA extraction, PCR and RTqPCR experiments

Total RNA was extracted from T24 cells using the TriReagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and 1 µg of each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, USA) following the manufacturer's protocol. The reactions were incubated as follows: 25° C for 10', 37° C for 120' and 85° C for 5', then their volume was brought to 50 µl with DEPC-treated water. Semi quantitative PCR experiments were performed using the Hot Star Taq Master Mix Kit (Qiagen, Hilden, Germany) in the Veriti 96 Well Thermal Cycler (Applied Biosystems Inc., Foster City, USA). 1 µl of cDNA for each sample was amplified with the same primer pairs that were designed to be used in the RTqPCR experiments. Thermal cycling conditions were as follows: 95° C for 10', followed by 40 cycles of 15'' at 95° C, 15'' at 60° C, 15'' at 72° C. The PCR products were analyzed on a 3% [w/v] TBE agarose gel and the 100 bp DNA Ladder (New England Biolabs, Ipswich, USA) was used as a molecular-weight size marker. RTqPCR analysis of cDNA samples was performed using the Power SYBR Green PCR Master Mix 2x (Applied Biosystems Inc., Foster City, USA) and the ABI PRISM 7700 Sequence Detector (Applied Biosystems Inc., Foster City, USA). We used the $2^{-\Delta\Delta Ct}$ method (Livak method) for relative quantification of gene expression. β-Actin was utilized as the housekeeping gene. The following list of primers was used: β -Actin F: 5'-GCGAGAAGATGACCCAGATC-3', R:5'-GGATAGCACAGCCTGGATAG-3' (77bp), BCL2 F:5'-GGCTGGGATGCCTTTGTG-3', R: 5'-GCCAGGAGAAATCAAACAGAGG-3' (64bp), BAX F: 5'-GCCCTTTTGCTTCAGGGTTT-3', R: 5'-TCCAATGTCCAGCCCATGAT-3' (356 bp), BIRC5 (Survivin) F: 5'-ATTCGTCCGGTTGCGCTTTCC-3', R: 5'-CACGGCGCACTTTCTCCGCAG-3' (162 bp), CTNNB1(β-Catenin),F:5'-AAAATGGCAGTGCGTTTAG-3',R:5'-TTTGAAGGCAGTCTGTCGTA-3' (144 bp).

Cytosol extraction

T24 cells were untreated, or treated with 2.5 µg/ml XVX, 50 µg/ml BC, 2.5 µg/ml XVX+50 µg/ml BC for 24h, then the Petri dishes were put in ice. The medium was removed and the cells were washed twice with 4 ml of Phosphate Buffered Saline (PBS) 1x. 100 µl of Cell Lysis Buffer (BioVision, Milpitas, USA) were added to each Petri dish and the cells were scraped for 10 min, then the lysates from each sample were recovered and transferred into fresh 1.5 ml tubes. The samples were put at -80° C for 30 min, than were unfrozen in ice and sonicated for 25 min at room temperature in a water-sonication bath. Samples were centrifuged at 12000 x g at 4° C for 10 min, than the supernatants (the cytosols) were recovered and put into fresh 1.5 ml tubes. Protein concentration of each cytosol was assessed through Bradford assay (BioRad Laboratories, Hercules, USA), measuring the absorbance values at λ=595 nm.

Determination of caspase 3 and caspase 8 activity

Caspase 3 and caspase 8 activities were assessed through the Caspase Colorimetric Assay Kit (BioVision, Milpitas, USA) according to the manufacturer's instructions. 100 µg of proteins from untreated or treated T24 cells were incubated with caspases reaction buffer (25 mM Hepes pH 7.4, 50 mM NaCl, 0.05% CHAPS, 0.5 mM EDTA, 5% glycerol, 5 mM DTT), then the substrate (200 µM final concentration) DEVD-pNA for caspase 3 or IETD-pNA for caspase 8 was added and the samples were transferred in a 96-wells microplate. All samples were incubated at 37° C for 2h. The absorbance, representing the activity of caspases, was measured at λ=405 nm in a microplate reader (BioRad Laboratories, Hercules, USA).

Statistical analysis

Results were expressed as mean ± SD and the statistical significance was assessed by one-way ANOVA, using PRISM 5.1 (Graph pad Software, La Jolla, USA). The results were considered statistically significant when $p<0.05$.

Results and discussion

Purification of betalains

The structural formula of betalains are shown in Fig. 1. Betalains, purified from BVr roots, were isolated by DEAE

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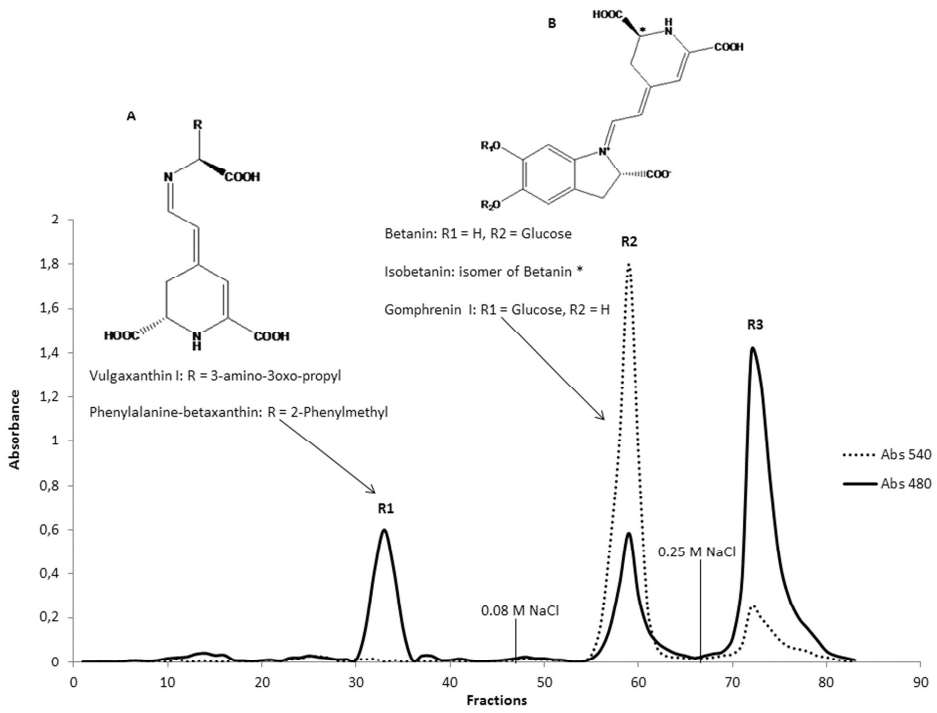

Fig. 1. Chromatographic profile showing the separation of the R1 peak, containing the BX fraction, from the R2 peak, containing the BC fraction. Chemical structures of (A) vulgaxanthin I and phenylalanine-betaxanthin, identified in the R1 peak; (B) betanin, isobetanin and gomphrenin I, identified in the R2 peak. The separation of BX from BC was performed by means of a stepwise elution with 0.08M NaCl solution. The R3 peak, eluted with 0.25 M NaCl solution, contained mainly neobetanin and phenolic acids and was not used in the experiments. Absorbance values at λ=480 nm and λ=540 nm, were used for detecting BX and BC, respectively.

chromatography in the three fractions R1, R2 and R3 (Fig. 1), desalted and analyzed by HPLC-DAD-MS analysis for their composition, as reported in Table 1. In the R1 fraction, vulgaxanthin I and phenylalanine-betaxanthin were identified (Fig. 1A). In the R2 fraction, a mixture of four betacyanins was found: betanin, isobetanin, gomphrenin I and isogomphrenin I (Fig. 1B). Gomphrenin I and isogomphrenin I are known as structural isomers of betanin [28]. In the R3 fraction, neobetanin, 2-decarboxy neobetanin and 2-decarboxy-2,3-dehydro-neobetanin were identified, but *p*-coumaric acid was detected as well. Since the R3 fraction was not composed of only betalains, it was excluded from the cytotoxicity experiments in T24 bladder cancer cells.

Effect of betalains and XVX on T24 cancer cell proliferation

The anti-proliferative effect of betalains and XVX fractions on T24 urinary bladder cancer cells was tested by SRB assay in dose response and time-course experiments (Fig. 2).

Table 1. Characterization and purification of the betalains of R1, R2 and R3 fractions from BVr.

Peak identification label	λ max (nm)	HPLC retention time (min)	m/z $[m+H]^+$	Compounds
R1 (BX)	471	8.87	359	Phenylalanine betaxanthin
	471	9.90	340	Vulgaxanthin I
R2 (BC)	534	16.98	551	Betanin
	534	17.43	551	Isobetanin
	534	18.35	551	Gomphrenin I
	534	19.55	551	Isogomphrenin I
R3	317	13.13	357	*p*-coumaric acid
	470	15.48	549	Neobetanin
	419	18.10	505	2-Decarboxy-neobetanin
	460	23.55	503	2-Decarboxy-2,3-dehydro-neobetanin

Compounds were identified by HPLC/MS analysis of the fraction peaks, eluted using the DEAE FF column chromatography and G10 desalting column. As R1 contained exclusively betaxanthins and R2 betacyanins, these bioactive mixtures were labeled as BX and BC, respectively, in the cytotoxicity studies. Since R3 fraction was composed by a heterogeneous mixture of phenolic acids and degraded betalains, it was discarded.

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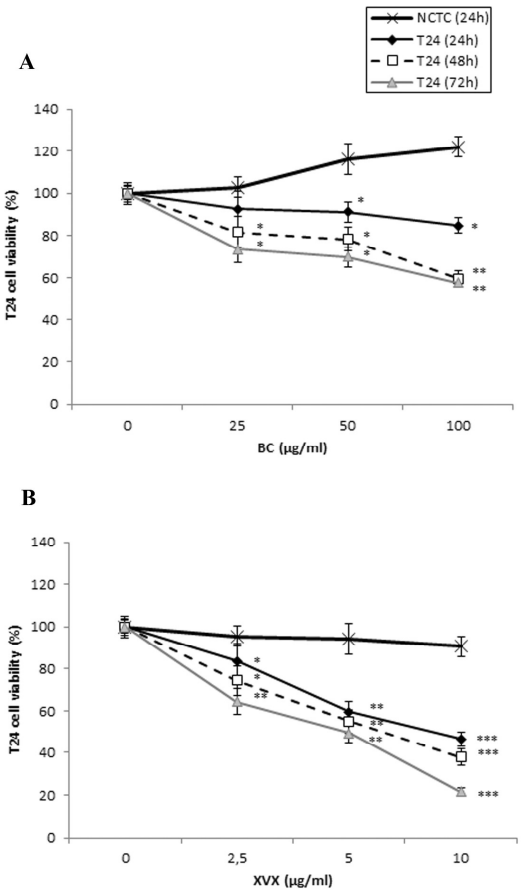

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g. 2. Cytotoxic effects of betacyanins (BC) and vitexin-2-O-xyloside (XVX) in T24 cancer cells after 24, 48 and 72 h treatments. The % of cells viability after (**A**) BC and (**B**) XVX treatment is shown. The same phytochemical treatments were performed on NCTC 2544 normal human cells for 24 h (**A**, **B** thick black line). Three replicate experiments with three samples analyzed for each replicate were performed. *p<0.05, **p<0.01, ***p<0.001.

The R1 fraction, containing BX, showed no anti-proliferative effect during the investigated period (*data not shown*), while the R2 fraction, containing BC, showed a dose-response antiproliferative effect (Fig. 2A), with an IC50 of 99.8±19.9 µg/ml at 72 h. Moreover, the combination of BX and BC did not show a synergistic or antagonistic effect in the induction of T24 cancer cell mortality during the time course of cytotoxicity experiments (Table 1, Supplementary).

XVX showed a remarkable dose response anti-proliferative effect (Fig. 2B), which increased from 24 to 72 h. The XVX IC_{50} at 72 h was 5.1±0.5 µg/ml (8.8±0.8 µM).

Moreover, the same concentrations of XVX and BC, which were individually able to reduce cell viability of T24 human bladder cancer cells, did not show any significant cytotoxic effect on normal human skin NCTC 2544 keratinocytes over a 24h treatment period (Fig. 2A, B).

To evaluate the effects derived from possible interactions between the two phytochemicals on T24 cell viability, experiments with XVX+BC combination were performed. The individual concentrations of XVX (2.5 µg/ml) and BC (50 µg/ml), showing the lowest statistically significant cytotoxic effect, were combined and tested for cytotoxicity at 24, 48 and 72h. We found that the combination XVX+BC showed the highest synergistic effect at 24h (Table 2). Moreover, the XVX+BC treatment showed no effect on the proliferation rate of NCTC 2544 normal human cells after 24h (*data not shown*).

The combination of XVX and BC induces apoptosis in T24 cancer cells

We investigated the ability of XVX and BC, used individually and in combination, to induce apoptosis in T24 cancer cells, after 24h of treatment. FACS analysis showed that XVX alone (p=0.003), BC alone (p=0.038) and the combination XVX+BC (p=0.002) were all able to increase the percentage levels of apoptotic T24 cells, compared to the control (Fig. 3A), with 13.9±2.2% for XVX, 3.7±1.1% for BC and 16.9±2.8% for XVX+BC phytochemical treatments.

It has already been shown that XVX inhibits the proliferation of colon cancer cells, through the increase in cleaved and active caspase 9 and caspase 3 levels, with induction of intrinsic apoptosis [19]. To better understand the apoptosis exerted by XVX and BC in T24 cells, the caspase 3 activity was evaluated for both compounds, used alone or in combination. As shown in Fig. 3B, XVX treatment induced an extremely significant (p<0.001) increase of 893±89% in the activity levels of caspase 3 and BC treatment induced a significant (p=0.023) increase of 153±7% in caspase 3 activity.

Table 2. T24 cells mortality induced by individual and combined phytochemical treatments, and synergy evaluation at 24, 48 and 72 h.

Treatments	Compounds	Measured mortality (%)	Expected Mortality (%)	Measured/Expected ratio
24 h	XVX	16.3±1.6		
	BC	13.9±0.7		
	XVX+BC	58.2±2.8	30.2±2.7	1.93±0.16**
48 h	XVX	25.7±2.1		
	BC	22.2±1.7		
	XVX+BC	65.1±5.6	47.9±3.8	1.36±0.17*
72 h	XVX	35.7±3.5		
	BC	30.6±2.3		
	XVX+BC	62.5±3.9	66.3±4.8	0.94±0.14

XVX: 2.5 µg/ml; BC: 50 µg/ml; *p<0.05, **p<0.01, when compared with the additive effect (1.0±0.1).

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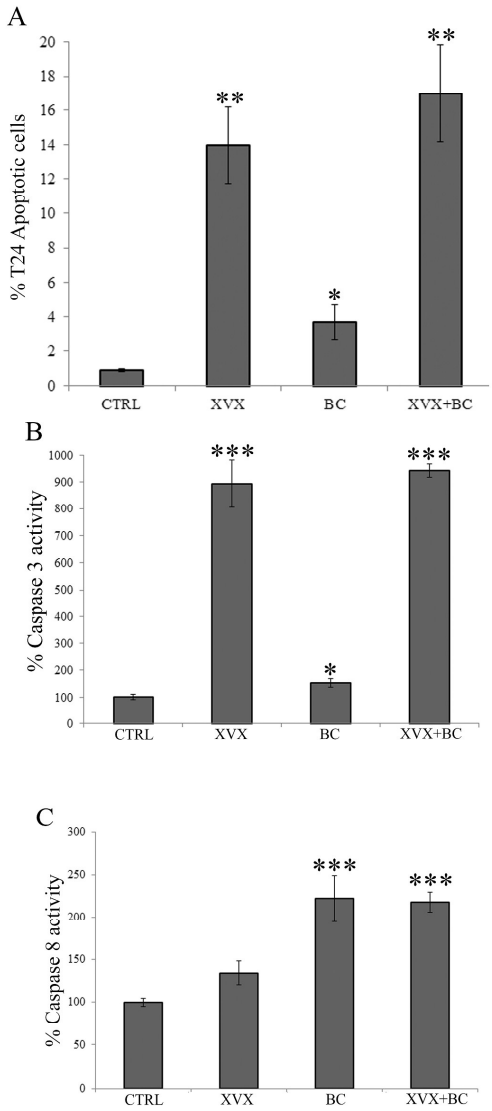

Fig. 3 Evaluation of apoptosis induction, caspase 3 and caspase 8 activity levels, induced by vitexin-2-O-xyloside (XVX), betacyanins (BC) and their combination in T24 cancer cells. **(A)** Cells were untreated (CTRL) or treated for 24 h with XVX (2.5 µg/ml), BC (50 µg/ml) or XVX+BC (2.5 µg/ml XVX+50 µg/ml BC). Data were expressed as % apoptotic cells. Two replicate experiments with two samples analyzed for each replicate were performed. (**B, C**) Cells were untreated (CTRL) or treated for 24h with XVX (2.5 µg/ml), BC (50 µg/ml) or XVX+BC (2.5 µg/ml XVX+50 µg/ml BC). Data were expressed as % of caspase 3 activity levels (**B**) or caspase 8 activity levels (**C**). Two replicate experiments with two samples analyzed for each replicate were performed. *p<0.05; **p<0.01; ***p<0.001.

XVX+BC treatment also showed an extremely significant (p<0.001) increase of 944±24% in the activity levels of caspase 3. These data were consistent with those obtained with the FACS analysis. From the histograms (Fig. 3A) it appears that XVX was mainly responsible for the apoptosis induction in T24 cancer cells, although the contribute of BC remained significant.

As shown in Fig. 3C, BC treatment was able to induce an extremely significant (p<0.001) increase of 222±27 % in caspase 8 activity levels, while XVX was not able to induce a significant rise (p=0.096). Moreover, the XVX+BC treatment induced an increase of 218±12% in caspase 8 activity levels, highlighting the role of BC in the activation of extrinsic apoptosis in T24 cancer cells.

The combination of XVX and BC modulates the expression levels of BAX, BIRC5 and CTNNB1

To shed light on the genes, that regulate the apoptosis induction mediated by XVX and BC treatments, semi-quantitative PCR experiments in T24 cells were performed.

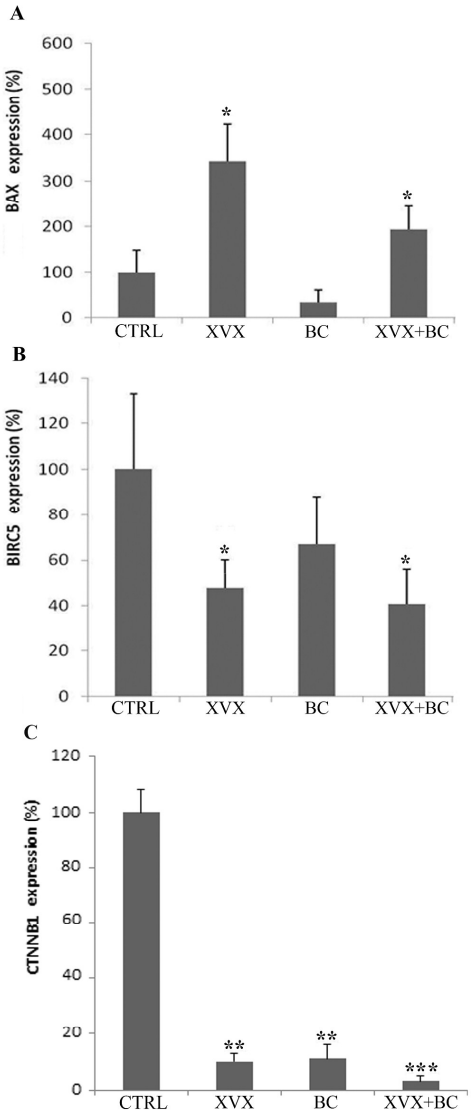

Fig. 4 Evaluation of the effects of vitexin-2-O-xyloside (XVX) and betacyanins (BC) and their combination (XVX+BC) on BAX, BIRC5 and CTNNB1 expression levels in T24 cancer cells using the RTqPCR assay. BAX (**A**), BIRC5 (**B**), CTNNB1 (**C**) mRNA levels were measured and normalized to the mRNA levels of the housekeeping gene β-actin. RNA was extracted from untreated cells (CTRL) and from T24 cancer cells treated for 24h with XVX (2.5 µg/ml), BC (50 µg/ml), or XVX+BC (2.5 µg/ml XVX+50 µg/ml BC). Three replicate experiments with three samples analyzed for each replicate were performed. *p<0.05, **p<0.01, ***p<0.001.

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Our results showed no significant changes in the expression levels of BCL2 gene (Figure 1, Supplementary), while a modulation in the expression levels of BAX, BIRC5 (Survivin) and the pro-survival gene CTNNB1 (β-Catenin) was observed and RTqPCR experiments were performed to quantify the changes in the expression levels of BAX, BIRC5 and CTNNB1, after 24 h of XVX, BC or XVX+BC treatments. The results of the RTqPCR analysis are shown in Fig. 4.

XVX and XVX+BC treatments were able to significantly increase the expression levels of pro-apoptotic BAX (p=0.012; p=0.042, respectively), as shown in Fig. 4A, without influencing the expression levels of anti-apoptotic BCL2, leading to an increase in the BAX/BCL2 mRNA ratio. The increase in the BAX/BCL2 mRNA ratio is not fully representative of changes in protein ratio. However, our results showed that the changes in the BAX mRNA levels are remarkable (Fig. 4A) and suggest an increase in the Bax/Bcl2 protein ratio, which can lead to apoptosis induction. The increase in BAX/BCL2 mRNA ratio has been recently used to explain the pro-apoptotic effect of flavanols extracted from Japanese quince fruit on prostate and breast cancer cell lines [29].

As shown in Fig. 4B, BIRC5 (Survivin) expression levels were significantly reduced by XVX (p=0.045) and XVX+BC treatments (p=0.044). Survivin is a member of the Inhibitor of Apoptosis Proteins (IAP) family, which protects the cells from the triggering of the apoptotic process. The pro-survival role of survivin consists in interfering with the cleavage and activation of caspase 9, the upstream initiator of the intrinsic mitochondrial pathway of apoptosis [30]. Hence, survivin downregulation can overexpose the T24 cancer cells to the cytotoxic effects of the phytochemicals, as it was previously observed for curcumin [31].

Moreover, as shown in Fig. 4C, 2.5 µg/ml XVX, 50 µg/ml BC and their combination were all able to significantly reduce the expression levels of CTNNB1 gene (p=0.002 for XVX, p=0.003 for BC and p<0.001 for XVX+BC). Our results indicate that the XVX+BC treatment showed a marked effect in downregulating CTNNB1 expression levels. It has already been shown that the reduction of the β-catenin expression levels, obtained through the RNA interference technique, can cause a relevant reduction of about 50% in the proliferation rate of T24 bladder cancer cells [32]. So, we hypothesized that the reduction of the expression levels of the pro-survival gene CTNNB1, exerted by the combination of XVX and BC, could justify the reduction of the proliferation rate which cannot be attributed to apoptosis mechanisms. Although BC was not as cytotoxic as XVX, as shown by their IC_{50} values in T24 cancer cells, the specific BC contribution, in combination with XVX, was associated with the activation of the extrinsic apoptotic pathway through a remarkable increase in caspase 8 activity levels (Fig. 5), which in turn induced a 1.5 fold increase in caspase 3 activity levels. However BC were not able to downregulate BIRC5 or to upregulate BAX expression levels, showing that they cannot activate the intrinsic pathway of apoptosis in p53 mut T24 cancer cells, as shown by Nowacki et al. [16] in the p53 mut HT29 cancer cells.

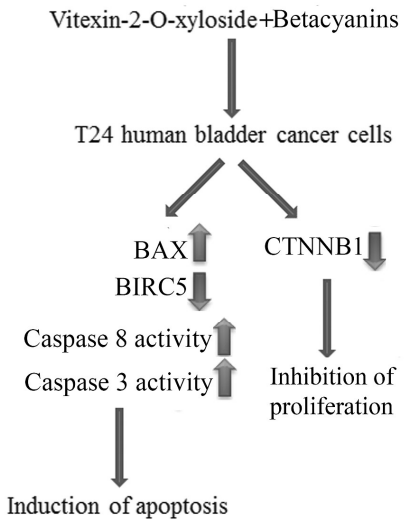

Fig. 5. Molecular pathways of apoptosis induction and inhibition of proliferation mediated by the combination of vitexin-2-O-xyloside+betacyanins in T24 bladder cancer cells. The combined treatment is able to increase the expression levels of pro-apoptotic BAX, downregulate the anti-apoptotic BIRC5 and increase the activity levels of caspases 8 and 3, leading to apoptosis induction. Moreover, the combined treatment downregulates the expression levels of the pro-survival transcription factor CTNNB1 (β-catenin), causing the inhibition of proliferation of T24 cancer cells.

XVX induced a relevant increase in caspase 3 activity levels, triggered by the overexpression of the pro-apoptotic gene BAX and the downregulation of the anti-apoptotic gene BIRC5 (Survivin). Both events are linked to the induction of the intrinsic pathway of apoptosis, leading to activation of the effector caspases [33].

In T24 cancer cells treated with our XVX+BC mixture, we hypothesized that BC activate only the extrinsic apoptotic pathway, whereas XVX is able to induce the intrinsic apoptosis, regardless the p53 mutations, because it probably triggers a ROS production, which damages directly the mitochondria [19].

The combined anticancer effect exerted by XVX and BC in T24 cells was caused by both the augmented induction of apoptosis (intrinsic and extrinsic), and the downregulation of the pro-survival gene CTNNB1, an event that can lead to relevant antiproliferative effect (Fig. 5), as already demonstrated in T24 bladder cancer cells [32].

Conclusions

In this work, we demonstrated that BC increase the ability of XVX to reduce the proliferation rate of T24 bladder cancer cells, without harming normal human NCTC 2544 cells. XVX and BC represent a bioavailable [4,34], mixture of phytochemicals to be used in combination for effective chemoprevention of bladder cancers. In fact, XVX activated the intrinsic apoptotic

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pathway and BC the extrinsic one, showing, when used in combination, a remarkable synergistic effect, very likely justified by the downregulation of the CTNNB1 gene.

Since actually the synthetic leads for BC and XVX are not available, the purification from plants is the sole way to obtain this cocktail of phytochemicals. XVX was isolated from seeds of one cultivar of BVc, obtained by a local company. The BC purification method was standardized starting from red beetroot cultivated by us under organic conditions. The mixture of betanin, isobetanin and two gomphrenin isomers is obtained under highly reproducible conditions. The isolation methods, together with the bioactivity evaluation, as well as the molecular mechanisms here described, document the connection between food and disease treatment [35].

This chemopreventive cocktail warrants further investigation in a second phase of studies, in which the *in vivo* anticancer activity of XVX and BC could be evaluated.

Conflict of interest

All the authors have no conflict of interest to declare.

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Abbreviations

BC, Betacyanins
BVc, *Beta vulgaris* var. *cicla* L.
BVr, *Beta vulgaris* var. *rubra* L.
BX, Betaxanthins
FACS, Fluorescence Activated Cell Sorting
XVX, Vitexin-2-O-xyloside

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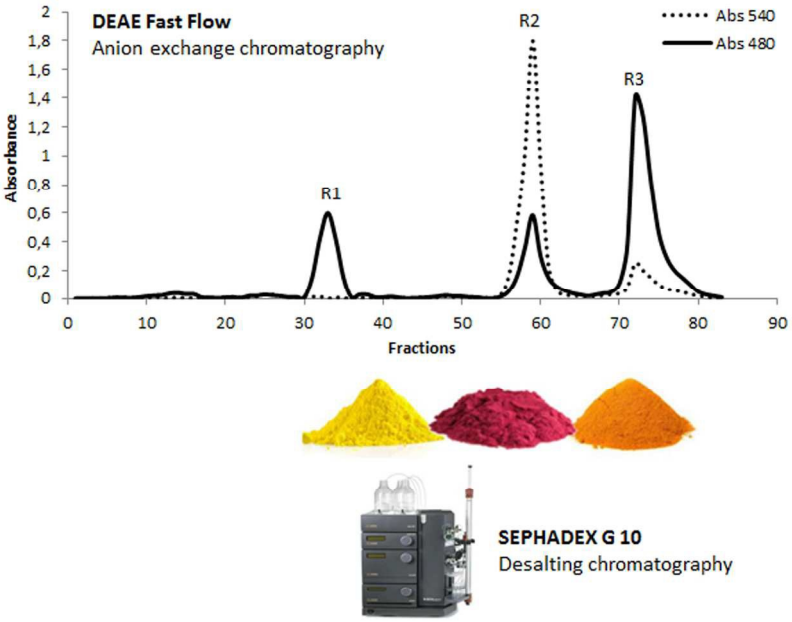

Innovative betacyanin purification method and treatment of T24 urinary bladder cancer cells in combination with vitexin-2-O-xyloside