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Betalains increase vitexin-2-O-xyloside cytotoxicity in CaCo-2 cancer cells

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Betalains increase Vitexin-2-O-xyloside cytotoxicity in CaCo-2 cancer cells

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Abstract

~~Phytochemicals with anticancer activity are present in *Beta vulgaris*. We purified~~ Vitexin-2-O-xyloside (XVX) from *Beta vulgaris* var. *cicla* L. (BVc) seeds, betaxanthins (R1) and betacyanins (R2) fractions from *Beta vulgaris* var. *rubra* L. (BVr) roots~~. Phytochemicals~~ were combined and tested for cytotoxicity in CaCo-2 colon cancer cells. XVX was the most cytotoxic molecule, but the combination of XVX with R1 and R2 significantly protracted its cytotoxicity. Cytotoxicity was mediated by ~~an~~ intrinsic apoptotic pathway, as shown by an increase in Bcl2-like protein 4, cleaved Poly ADP-Ribosyl Polymerase 1 and cleaved Caspase 3 levels with a parallel decrease in anti-apoptotic protein B-cell leukemia/lymphoma 2 levels. R1 and R2, used alone or coupled, ~~were also able to~~ reduced oxidative stress triggered by H_2O_2 in CaCo-2. ~~Moreover,~~ Betalains dampened Cyclooxygenase-2 and Interleukin-8 mRNA expression after Lipopolysaccharide induction in CaCo-2, showing an anti-inflammatory action. Our results support the use of ~~an efficient nutraceutical~~ a cocktail of R1, R2 and XVX as a chemopreventive tool against colon cancer.

Key words: *Beta vulgaris*, vitexin-2-O-xyloside, betalains, antitumoral activity, CaCo-2 cancer cell line, nutraceutical products.

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1.Introduction

Swiss chard (*Beta vulgaris* var. *cicla* L.; BVc) and beetroot (*Beta vulgaris* var. *rubra* L., BVr) are vegetables of the *Chenopodiaceae* family, which have been part of the traditional western diet for ages~~. These vegetables have been used in folk medicine as a therapy for tumors of the digestive system, breast, prostate and uterus~~ (Ninfali & Angelino, 2013).

From a nutritional standpoint, BVc is one of the most powerful antioxidant vegetable (Ninfali & Bacchiocca, 2003), due to the presence of a class of flavonoids derived from apigenin, namely vitexin, vitexin-2-O-glycoside, vitexin-2-O-rhamnoside and vitexin-2-O-xyloside (XVX). All these molecules, with the exception of XVX, are commercially available. XVX has been purified from hawthorn leaves (Ma, Liu, Xu, Yu, Zhang, & Liu, 2010) or BVc seeds (Gennari et al., 2011) and its cytotoxicity in cancer cell lines has been previously demonstrated (Ma et al., 2010). In addition, a synergistic cytotoxic effect of XVX in combination with epigallocatechin-3-gallate and isothiocyanates in colon cancer cell lines has also been reported (Papi et al., 2013).

The red beetroot (BVr) extract possesses relevant antioxidant activity (Kugler et al., 2007) thanks to a class of molecules grouped under the name of betalains, derived from betalamic acid (Stintzing et al., 2004). Betalains are subdivided into red-violet betacyanins and yellow betaxanthins: with the former showing a higher antioxidant activity than the latter, as shown by both ABTS and FRAP methods (Castellar, Solano, & Obon, 2012; Feugang, Konarski, Zou, Stintzing, & Zou, 2006; Kugler et al., 2007). After studying betalains and other natural food colorant, Reddy, Alexander-Lindo, & Nair (2005) showed that betacyanins inhibited lipid peroxidation and growth of several tumor cell lines. However, when combined with antocyanins, betacyanins were not able to inhibit the tumor cell growth, as well as the pro-inflammatory cyclooxygenase-2 enzyme, as the two molecules proved to be antagonistic (Reddy et al., 2005). Two isoforms of cyclooxygenase (COX) have been identified and referred to as COX-1 and COX-2. While COX-1 is constitutively expressed in tissues, COX-2 is

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an inducible isoform and its expression is up-regulated by cytokines, mitogens, growth factors and carcinogens (Schneider & Pozzi, 2011). COX-2 levels have been reported to increase dramatically in ulcerative colitis (Sheng, Shao, Morrow, Beauchamp, & Dubois, 1998) and colorectal cancer (CRC) (Hardy, Meltzer, & Jankowski, 2000). Genetic (Strillacci et al., 2010) and pharmacological (Schneider et al., 2011) inhibition of COX-2 has resulted in decreased incidence of primary and metastatic tumor growth, making COX-2 and its products ideal targets for anti-neoplastic therapy. Many natural compounds are able to inhibit COX-2 activity: resveratrol, epigallocatechin-3-gallate and tyrosol from olive oil extract (Schneider et al., 2011). Although most of the pro-tumorigenic activity of COX-2 is attributed to the generation of prostaglandin E2 (PGE2), other COX-2 derived products may also affect tumor development: prostaglandin F2α (PGF2α) and thromboxane (TxA2). High levels of PGE2 can increase Interleukin-8 (IL-8) mRNA and protein production in colonic epithelial cells, contributing to the inflammation process in the gastrointestinal tract (Yu & Chadee, 1998). IL-8 is the principal inflammatory cytokine involved in the inflammation process, which can lead to the development of colon cancer (Lee et al., 2012). It is therefore important for human health to reduce inflammation by means of natural compounds contained in foods, such as betalains from red beetroot.

The betalain content of five red beetroot varieties was determined by Gasztonyi, Daood, Hájos, & Biacs (2001), who concluded that the red beet variety *Detroit* is one of the richest in pigment. Several medium pressure liquid cromatography ~~(MPLC)~~ methods have been used for purification of betalains from red beetroot (Račkauskienė, Pukalskas, Venskutonis, Fiore, Troise, & Fogliano, 2015). Betalains can be separated into betacyanins and betaxanthins in different stationary phases such as hydrophobic interactions and anion exchange resins (Stintzing, Conrad, Klaiber, Beifuss, & Carle, 2004). Higher purification grade and complete separation of betalains have been obtained using preparative high performance liquid chromatography (HPLC) techniques, but with low yields

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(Kugler, Stintzing, & Carle, 2004). The aim of this paper was to develop a simple scale up method for the purification of definite fractions of betacyanins and betaxanthins and to test their cytotoxic effect individually and in combination with XVX in CaCo-2 colon cancer cells, which are characterized by mutated TP53 gene and over expression of multi-drug resistance (MDR) proteins (Piccirillo, Filomeni, Brune, Rotilio, & Ciriolo, 2009).

2. Materials and methods

2.1 Chemicals

Ethanol and ethyl-acetate (analytical grade) were purchased from VWR International (Radnor, USA). Fluorescein sodium salt, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), DMEM culture medium, foetal bovine serum (FBS), antibiotics, trypsin, water (LC-MS grade), acetonitrile (LC-MS grade) and formic acid (LC-MS grade) from Sigma-Aldrich (St. Louis, USA). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Polysciences, Inc. (Warrington, USA). Lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, USA) was used at 1 µg/ml final concentration. This concentration did not affect cell viability (data not shown).

2.2 Plant material

Seeds of BVc were obtained from Suba Seeds Company S.r.l. (Longiano, Italy). Seeds were identified by a lot number and the company ensured the quality and the homogeneity of the product. Seeds were stored at room temperature in a dry dark environment. Red beetroots (cv. *Detroit*) were cultivated in Castel Cavallino, Urbino (Italy). The beetroots were harvested at a stage of commercial harvest maturity, washed and 100 g of roots were used for the extraction of the betalains.

2.3 Preparation of XVX

The compound was isolated from seeds of BVc (cv. *Bietola verde da taglio).* The extract from seeds was prepared by solid-liquid extraction with 70% ethanol in a Timatic extractor (Tecnolab S.r.l.,

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Spello, Italy) and liquid-liquid extraction with ethyl-acetate, as previously reported (Gennari et al., 2011). Two chromatography columns were then applied: a Diaion HP-20 (Sigma-Aldrich, St. Louis, USA) and a Davisil C18 (ALLTECH, Milan, Italy), performed by means of a LC-Chromatograph AKTA Purifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the elution of XVX at 336 nm. The elution phase of the Diaion HP 20 was 30% ethanol, isocratic; an elution gradient composed by formic acid 0.05% (buffer A) and ethanol 60% (buffer B) was used for Davisil C18. Three phenol fractions were obtained: P1, P2 and P3. P1 did not contain XVX and was discarded, whereas P2 and P3, containing 95% and 70% of XVX, respectively, were stored. For all the experiments performed on cells, we used the P2 fraction only.

2.4 Purification of Betalains

The beetroots were grated and extracted (1:10 w/v) with 70% ethanol in 10 mM Na-acetate buffer pH 5.0 for 2 h under stirring at +6 °C. The extract was filtered and concentrated by rotavap until reaching 10% of the initial volume, then subjected to liquid-liquid extraction with ethyl acetate. The remaining aqueous extract containing the betalains was lyophilized and stored at –80°C. Betalain subfamilies were isolated by means of a DEAE Sepharose fast flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column chromatography, performed by means of the LC-Chromatograph AKTA Purifier 10, ~~(GE Healthcare Bio-Sciences AB, Uppsala, Sweden)~~ as follows. The lyophilized extract (5 g) was suspended in 10 mM Na-phosphate buffer pH 7.5 and loaded onto the column (5.0 × 30 cm). The separation of the betaxanthins from the betacyanins was achieved with a NaCl gradient from 0 to 0.5 M in 10 mM Na-phosphate buffer pH 7.5. Betalains were assayed by reading the absorbance at 536 nm and 477 nm and using an extinction coefficient at 1% i.e. $E^{1cm}_{1\%}$=1120 at 536 nm for betacyanins and $E^{1cm}_{1\%}$=750 at 477 nm for betaxanthins (Chethana, Nayak, & Raghavarao, 2007; Nilsson, 1970). **Figure 1A** shows the chromatographic profile of the DEAE Sepharose fast flow column with the gradient applied for the elution of betalains. Three fractions were obtained and

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labeled as R1, R2 and R3. After lyophilization, R1 was a yellow powder with maximal absorbance at 477 nm. R2 was a red powder with maximal absorbance at 536 nm, corresponding to betacyanins. R3 was found as a yellow pigment with spectra λ max at 330, 399 and 477 nm which indicated the presence of a betaxanthin together with flavonoids (Vulic et al., 2013). Since the components of R3 were unknown it was not used for cells treatments, waiting for its complete identification. Therefore, we proceeded with HPLC - diode array detector (DAD) analysis of R1 and R2, in order to characterize them for the cells treatments experiments.

2.5 Analysis by HPLC-DAD

Samples of the lyophilized betalains peaks R1 and R2 were dissolved into the mobile phase and analyzed by HPLC-DAD as reported (Cejudo-Bastante, Chaalal, Louaileche, Parrado, & Heredia, 2014). The HPLC was equipped with a Spherisorb ODS2 column (Waters Corporation, Milford, USA), with the dimensions of 4.6 × 250 mm; the particle size was 5 µm. Standard and sample volumes were 20 µl. Separation of betalains was performed using 1% formic acid in water (solvent A) and methanol (solvent B). A linear gradient was applied starting from 0 to 10% B in 20 min, then from 10 to 30% B in 10 min, then from 30 to 100% B in 5 min. To re-establish the initial conditions, a linear gradient from 100% B to 100% A was used during 5 min. Detection was performed at 536 nm and 477 nm for red-violet and yellow pigments, respectively. **Figure 1B** shows the HPLC chromatogram of the beetroot crude extract, that evidenced 4 distinct peaks. The upper inset of **Figure 1B** shows the HPLC chromatogram of R1 presenting a main peak. R2 was found to be composed mainly by three betacyanins with maximal absorbance at 536 nm (**Figure 1B**, lower inset). The main peaks in R1 and R2 were identified by HPLC-ESI-MS.

The HPLC analysis of the XVX was performed on the same equipment with a gradient consisting into 0.01% acetic acid in water (solvent A) and methanol containing 0.01% acetic acid (solvent B).

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The gradient was the following: from 0 to 60% solvent B in 45 min at 1 ml/min, then to 100% B in 5 min. XVX was identified using a purified standard available in our lab (Gennari et al., 2011).

2.6 HPLC-ESI-MS

A Series 200 Micro Pump HPLC system (Perkin Elmer, Norwalk, USA) was equipped with an API 150 EX (Applied Biosystems, Inc., Foster City, USA) mass spectrometer with an electrospray ionisation (ESI) source. The column and gradient were the same as those used for the analytical HPLC. The injection volume was 20 µl and the flow rate was 1ml/min, split to 0.2 ml/min to the ESI source. Compounds were identified analysing the m/z of molecular ions (ESI^+) and by comparing them with those found in the literature databases and UV/Vis spectra. The peak of R1 (**Figure 1B** upper inset) was identified by mass spectrometry (MS) analysis as vulgaxanthin I (Gasztonyi et al., 2001). The three peaks of R2 were composed of three betacyanins identified in the decreasing order as betanin, isobetanin, betanidin (**Figure 1B** lower inset).

2.7 Determination of antioxidant activity

Antioxidant capacity was measured by the Oxygen Radical Antioxidant Capacity (ORAC) method as previously reported (Ninfali, Chiarabini, & Angelino, 2014). The ORAC values were expressed as micromoles Trolox equivalents (TE)/g of dry beetroot fraction.

2.8 Cell culture

CaCo-2 colon cancer cell line was purchased from the American Type Culture Collection (ATCC, Rockville, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µM MEM non-essential amino acid solution and grown at 37°C in a humidified atmosphere with 5% CO_2. Media and supplements were purchased from Sigma-Aldrich (St. Louis, USA).

2.9 Sulforhodamine B (SRB) assay

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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 (10^4/well) were plated in 96-well plates and treated with XVX, R1 and R2 for 24, 48 and 72 hours. At the end of treatment, cell culture medium was removed, and the cells were fixed using 100 µl/well of 50% aqueous trichloroacetic acid for 1 h at 4 °C, rinsed with water several times and incubated for 30 min with 100 µl/well SRB solution (0.4%) (Sigma-Aldrich, St. Louis, USA). After rinsing with 1% acetic acid and solubilizing in 10 mM Tris for 20 min, the absorbance of each well was measured in a microplate reader (BioRad Laboratories, Hercules, USA) at 570 nm. The results were expressed as a percentage of cell mortality referred to control.

2.10 Synergy evaluation

Compounds were tested individually and mixed in pairs or in triple combination in the cell culture medium and the cytotoxicity was evaluated by the SRB assay. 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 antagonistic effect; a Measured/Expected Ratio = 1.0 ($p<0.05$) indicated an additive interaction (Papi et al., 2013).

2.11 Western blot

To determine Bcl2-like protein 4 (Bax), protein B-cell leukemia/lymphoma 2 (Bcl2), Caspase 3 and poly(ADP-ribose) polymerase-1 (PARP-1) protein levels, the CaCo-2 cancer cells were plated and treated with XVX, R1 and R2 individually and in the mixture. After treatment, cells were detached, collected by centrifugation at 300 g for 5 min, and the pellets were suspended in lysis buffer (20 mM

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Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 μM Na_3VO_4) and sonicated on ice, in the presence of protease inhibitors. Proteins (50 μg) from cell lysate were size fractioned in 12% SDS-polyacrylamide gel, and transferred to Hybond TM-C Extra membranes (GE Healthcare, Little Chalfont, UK) by standard protocols. After the blocking phase, the following antibodies were used according to supplier recommended concentrations: anti-Caspase 3 (Sigma-Aldrich, St. Louis, USA), anti-PARP-1 (Santa Cruz Biotechnology, Inc., Dallas, USA), anti-Bax (Applied Biosystems Inc., Foster City, USA), anti-Bcl2 (Sigma-Aldrich, St. Louis, USA). The primary antibodies were diluted 1:500 in TBS-5% milk and the respective peroxidase-conjugated secondary antibody was diluted 1:1000. The proteins were detected by luminol (GE Healthcare, Little Chalfont, UK). Bands were quantified by using densitometric images analysis software (Image Master VDS, Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Protein loading was controlled by actin (1:1000) (Sigma-Aldrich, St. Louis, USA) detection after stripping of membranes with stripping solution (Thermo Fisher Scientific, Waltham, USA). Values were normalised against the actin control and statistically evaluated.

2.12. Reactive Oxygen Species (ROS) assay

Generation of ROS in CaCo-2 cells was monitored by the conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein (DCF). DCFH-DA is transformed by cellular esterase into the reduced not fluorescent form DCFH, which, in the presence of ROS, is oxidized to highly fluorescent DCF. Cells were grown on coverslips ($2x10^4$/well) in 24 well plates and treated for 24 h with R1 and R2 individually and in combination and then incubated with 1 mM H_2O_2 for 1 h at room temperature. Control cells did not receive any treatment, whereas another sample was only treated with 1 mM H_2O_2 for 1 h as a positive control. After washing with PBS, all the samples were incubated in the dark for 20 min with 10 μM DCF-DA (Sigma-Aldrich, St. Louis, USA) dissolved in PBS. After incubation, the cells were rinsed three times

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with PBS and mounted with a solution of Hoescht (10 µg/ml) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (both from Sigma-Aldrich, St. Louis, USA). The samples were observed with an epifluorescence microscope (Nikon Instruments Europe BV, Amsterdam, Holland) equipped with filters for Hoescht and FITC. Cells showing a bright and intense fluorescence were counted as positive, whereas cells with no or low fluorescence were counted as negative. At least 3 fields for sample were analysed and 100-200 cells were counted. Results were expressed as % DCF fluorescent positive cells vs control.

2.13. RNA extraction and RTqPCR (Real Time quantitative PCR) for COX-2 and IL-8

CaCo-2 cells were all incubated with LPS (1 µg/ml) for 25 min and then were treated for 24h with XVX (25 µg/ml), (XVX), or R1 (0.35 µg/ml) (R1), or R2 (0.25 µg/ml) (R2), or R1+R2 (R1+R2) or XVX+ R1+ R2 (XVX+R1+R2) and results were compared to those of the cells that have not been treated with phytochemicals (LPS only).

Total RNA was extracted from CaCo-2 cells using TRIzol (Thermo Fisher Scientific, Waltham, USA) reagent. RNA (1.5 µg) of each sample was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's protocol. RTqPCR analysis of cDNA was performed using a fluorescent nucleic acid dye (SsoFast™ EvaGreen Supermix, BioRad Laboratories, Hercules, USA) in a CFX96 system (BioRad Laboratories, Hercules, USA). We used the $2^{-\Delta\Delta C_T}$ method for relative quantification of gene expression. β-Actin was used as the house keeping gene. The list of primers was the following: Actin: F:5'-GGCATCGTGATGGACTCCG-3', R-5'-GCTGGAAGGTGGACAGCGA-3' (192 bp); IL-8: F-5'-GCTTTCTGATGGAAGAGAGC-3', R-5'-GGCACAGTGGAACAAGGACT-3' (220 bp); COX-2: F:5'-CCTGTGCCTGATGATTGC-3',R:5'-CTGATGCGTGAAGTGCTG-3' (162 bp).

2.14 Statistical analysis

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Experiments were replicated three times with three samples analyzed per replicate. Results were expressed as mean ± SD of nine obtained values. Statistical significance was assessed by one-way ANOVA, using PRISM 5.1 (GraphPad Software, La Jolla, USA). The results have been considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1 Composition of R1 and R2

The composition of R1 and R2 is summarized in **Table 1** and it has been determined through absorbance values, HPLC retention times and MS analysis. This table also shows the antioxidant activity, measured by means of the ORAC method, of the R1 and R2 fractions, together with the betalains yields obtained in the purification process.

The chromatographic profile of the DEAE FF column chromatography is shown in **Figure 1A**. The purification process was scaled up to 5 g of dry extract, dissolved into 40 ml of equilibrating buffer, loaded onto the column. Considering the total areas of the three peaks, it was determined that the betaxanthins of R1 represent the 11% of the sum of the peaks area and that the betacyanins of R2 represent the 61%. Since the R3 peak, accounting for 28% of the whole peaks area, was discarded for its content of flavonoids, ~~we were able to obtain,~~ for each 5 g of extract processed with the DEAE FF chromatography, 0.5 ± 0.1 g of lyophilized R1 and 3.5 ± 0.5 g of R2 were obtained.

The R2 fraction was that endowed of the highest antioxidant capacity, thus confirming data reported in literature regarding the higher antioxidant capacity of betacyanins, compared to betaxanthins (Kugler et al., 2007). As R1 was entirely composed by betaxanthins and R2 by betacyanins **(Table 1)**, these two fractions were utilized for in vitro cytotoxicity studies in CaCo-2 cancer cell line.

3.2 Antiradical activity of R1 and R2 as detected by DCF-DA assay

~~We induced~~ The oxidative stress was induced by H_2O_2, which elicits ROS production, that can be visualized by the DCF-DA

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assay. CaCo-2 cells were treated with R1 or R2 or R1 + R2 for 24 h, then 1mM H_2O_2 was administered for 1 h and the presence of ROS was evaluated by the DCF-DA assay **(Figure 2)**. Cells producing ROS showed intense green fluorescence and could be evaluated as positive. In untreated control cells, rare positive cells were detected (6 ± 3%), whereas in CaCo-2 cells only treated with H_2O_2, the percentage of positive cells increased to 71 ± 13% **(Figure 2)**. The positive cells percentage decreased to 6.2 ± 3.1% and 3.5 ± 1.4% when the CaCo-2 cells were pretreated with R1 or R2, respectively. Moreover, the fluorescent cells decreased to 2.4 ± 0.6% when pre-treated with R1 + R2 **(Figure 2)**. These findings showed a powerful antioxidant activity for R1 and R2, when used alone and in combination. The fact that R1 and R2 were able to reduce the oxidative damage, induced by H_2O_2 directly into the cells, shows that the betaxanthins and betacyanins contained in the R1 and R2 fractions are able to cross the membranes and exert their antioxidant activity inside the cells.

3.3 Cytotoxicity of XVX, R1 and R2 in Caco-2 cells

~~We first investigated~~ The cytotoxicity of the individual phytochemicals was investigated. CaCo-2 cells were incubated with various concentrations of XVX for 24, 48 and 72 h and SRB assay was performed. The dose-response cytotoxic effect of XVX in the 12.5-50 µg/ml range is shown in **Figure 3A**. XVX exhibited the greatest effect on CaCo-2 cells viability when used at 50 µg/ml. The IC_{50} value was found at 25 µg/ml after 24 and 48 hours of incubation. At 25 µg/ml XVX concentration, the effect on cells viability was more relevant at 24 and 48 h than at 72 h, when a significant decrease in cell mortality was observed ($p = 0.004$). The cell death decrease at 72 h could mean that the CaCo-2 cells have the ability to metabolize or extrude part of the XVX within 72 h. On the contrary, at 50 µg/ml XVX concentration, the cytotoxic effect increased over time with the maximum effect at 72 h ($p = 0.002$). Hence, at this concentration, XVX showed sustained strong cytotoxicity over the testing period and the cells showed a less efficiency in metabolizing and extruding higher concentrations of the drug. ~~were unable to metabolize or extrude the drug~~A hormetic effect was observed when CaCo-

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2 cells were incubated with increasing concentrations of R1 and R2 fractions In fact, cytotoxicity was more evident at lower concentrations, than at higher concentrations **(Figure 3B, Inset)**. Regarding the specific cytotoxic concentrations, the highest effect was observed at 0.35 µg/ml R1 and 0.25 µg/ml R2. The cytotoxic effect of individual R1 and R2 was analysed in time-course experiments. The effect of 0.35 and 0.25 µg/ml R1 and R2 in CaCo-2 cells incubated for 24, 48 and 72 h is shown in **Figure 3B**. The cytotoxicity of both R1 (p = 0.036) and R2 (p = 0.026) significantly decreased after 72 h of treatment, thus indicating that CaCo-2 cells are able to metabolize and/or extrude R1 and R2.

In order to evaluate a possible synergistic effect of XVX in combination with R1 or R2, ~~we used~~ the 25 µg/ml XVX concentration, corresponding to the IC_{50} value, was used in combination with 0.35 or 0.25 µg/ml R1 or R2, respectively. XVX combined with R1 (p = 0.003) or R2 (p = 0.007), exhibited, in both cases, a high and significant cytotoxic effect even after 72 h of treatment **(Figure 3C)**. In fact, the XVX + R1 combination induced 67.7 ± 7.9% cells mortality, while the XVX + R2 combination induced 68.9 ± 3.8%. These results showed that both R1 and R2 exhibited a synergistic cytotoxic effect when used in combination with XVX. The synergy was evaluated by calculating the ratio of the measured/expected cytotoxicity, which was 1.53 ± 0.15 for XVX + R1 and 1.54 ± 0.16 for XVX + R2 **(Table 1, Supplementary)**. The ability of betalains to enhance XVX cytotoxicity may stem from their capacity to hamper XVX catabolism or inhibit its expulsion from the cells, probably by modulating the Multidrug resistance (MDR) system (Eid, El-Readi, Eldin, Fatani, & Wink, 2013). ~~We also tested~~ The cytotoxic effect in CaCo-2 cells of the triple combination XVX + R1 + R2 at the same concentrations used for the above mentioned combinations was also tested. The triple combination was the most cytotoxic combination for CaCo-2 cells, especially when used in the 24 h treatment **(Figure 3C)**, when compared to XVX + R1 treatment ($p < 0.001$) and XVX + R2 treatment ($p<0.001$). These results highlight the ability of the triple combination to maintain cytotoxicity

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through 72 h, when the cells mortality was 85.6 ± 5.1% and the measured/expected ratio was 1.39 ± 0.14 **(Table 1, Supplementary)**.

These results prompted us to analyse the effects of single phytochemicals and the triple combination on the molecular targets involved in the induction of apoptosis in CaCo-2 cells, as previously demonstrated for XVX (Papi et al., 2013).

3.4 Apoptosis induction in Caco-2 cells

XVX, R1 and R2 used alone or in the triple combination were incubated for 24 h with the CaCo-2 cells and Bcl-2, Bax, Caspase 3 and poly(ADP-ribose) polymerase-1 (PARP-1) expression levels were evaluated. The effects of these phytochemicals on the expression of the above mentioned molecular targets, involved in the apoptotic process, are shown in **Figure 4**. Bcl-2 protein expression levels significantly decreased in the presence of R1 and XVX + R2 + R1, but not in the presence of R2 or XVX alone. Conversely, the Bax protein expression levels increased with individual concentrations of R2, as well as with the combination of XVX + R2 + R1, but not with the treatment with R1 or XVX alone. Cleaved PARP-1 levels significantly increased compared to the untreated cells, both with the individual concentrations of XVX, R2 and R1 as well as with the combination of XVX + R2 + R1. The levels of cleaved Caspase 3 increased significantly with individual treatments of XVX, R2 and R1 as well as with the triple combination **(Figure 4)**.

Overall, a down regulation of Bcl-2 was observed, together with an over expression of the pro-apoptotic Bax protein, with some individual compounds, but always with the triple combination. The Bcl2/Bax ratio decrease is considered as an indicator of the activation of intrinsic (mitochondrial) pathway of apoptosis (Khan, Adhami, & Mukhtar, 2008). The downstream apoptotic signaling molecules, represented by the cleaved Caspase 3 as well as the cleaved PARP-1, were consequently increased. Caspase 3 plays a central role in the execution phase of cell apoptosis as it is responsible of the proteolytic cleavage of PARP-1, a nuclear enzyme involved in DNA repair and stability, which

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is cut and then inactivated by Caspase 3. On the whole, our results show the activation of an intrinsic apoptotic pathway in CaCo-2 cells, which was triggered to different extents by the individual compounds XVX, R1 and R2, but mainly by their combination. This means that there is a potential chemopreventive effect exerted by the three nutrients. Moreover, since these molecules were shown to be largely bioavailable (Tesoriere et al., 2013), a systemic cytoprotective effect in the intestine, liver and peripheral tissues is also conceivable.

3.5 Transcriptional inhibition of COX-2 and IL-8

~~We performed~~ RTqPCR experiments were performed to evaluate the mRNA expression levels of IL-8 and COX-2, the pro-inflammatory markers which support the over-proliferation of colon cancer cells (Sheng et al., 1998; Suh, Afaq, Johnson, & Mukhtar, 2009). ~~We observed~~ Detectable COX-2 and IL-8 mRNA levels were observed only after treatment with LPS, a known compound able to induce inflammation.

XVX alone induced a mild decrease in COX-2 mRNA expression levels; R1 also prompted a slight decrease in mRNA expression, whereas R2 markedly reduced mRNA expression levels. R1 + R2 as well as the triple combination XVX + R1 + R2 were both able to significantly reduce the mRNA expression levels of COX-2 **(Figure 5A)**. Therefore, the R1 + R2 combination and also the triple combination XVX + R1 + R2 inhibited the expression of COX-2 and its product PGE2, which in turn is no longer able to induce the over expression of Bcl-2, the anti-apoptotic protein (Suh et al., 2009). Th series of events strengthens the cytotoxic effect of our phytochemicals in CaCo-2 colon cancer cells.

Moreover, since IL-8 is the principal cytokine involved in the inflammation process that can lead to colon cancer development (Lee et al., 2012), ~~we also assessed~~ whether our phytochemicals were able to regulate IL-8 mRNA expression levels was assessed. ~~We observed~~ A mild decrease in IL-8 mRNA

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expression levels after XVX treatment was observed; whereas R1, R2, R1+R2 and XVX+R1+R2 treatments were all able to significantly reduce IL-8 mRNA expression **(Figure 5B)**.

4. Conclusions

~~We improved~~ The present study describes an improved ion exchange column chromatography protocol for purification of gram quantities of betaxanthins and betacyanins from red beetroot, that were used for cytotoxicity studies alone and in combination with XVX, the main cytotoxic flavonoid from BVc seeds. Our results showed that XVX, R1 and R2 individual treatments exerted a cytotoxic effect in CaCo-2 tumor cell lines at 24 and 48 h. Cytotoxicity of R1 and R2 showed a hormetic pattern and ~~we observed~~ the highest cytotoxicity was observed when the fractions were used in the range of 0.25-0.35 µg/ml, which is a quite low concentration range. In contrast, XVX showed its IC_{50} cytotoxic activity at 25 µg/ml. The combinations of XVX + R1, XVX + R2 and XVX + R1 + R2 led to a significant synergistic cytotoxic effect in CaCo-2 cells, with the highest cytotoxicity observed at 72 h. In other words, the cytotoxic effect of the couples and the triple combinations increased and protracted over the time. In a previous study (Papi et al., 2013), ~~we demonstrated~~ it has been shown that XVX could be more effective in triggering cancer cell death when combined with other phytochemicals. In the present study, ~~we show~~ it has been shown that there is a synergism among XVX, R1 and R2 that could improve their individual cytotoxic effects,

A possible explanation for the increased cytotoxicity of the XVX+R1+R2 treatment in comparison with XVX alone lies in the ability of betalains to significantly reduce COX-2 and IL-8 mRNA expression levels. This inhibition leads to a down regulation of the anti-apoptotic protein Bcl-2. In fact, our results show that XVX alone is not able to reduce Bcl-2 levels, while the triple combination XVX+R1+R2 can significantly decrease it.

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~~We do not know~~ It is unclear at present if the cytotoxic effect is triggered either by the intact phytochemicals or by their metabolites and therefore studies are in progress in our laboratory to detect the presence of metabolic products in the cells and in the culture medium.

~~Our findings also highlight the powerful antioxidant capacity of the two betalain fractions, as already reported (Esatbeyoglu, Wagner, Schini-Kerth, & Rimbach, 2015; Tesoriere et al., 2013).~~ Our data also suggest that R1 and R2 and their combination may work efficiently as anti-oxidant agents against ROS compounds, even inside the cells, taking advantage of their remarkable ability to cross the cell membrane.

Both ORAC and DCF-DA assays demonstrated the antioxidant activity of R1 and R2, when used individually and in combination. H_2O_2 treatment triggered a robust ROS production, which was abolished when the cells were pre-treated with R1 and R2 for 24 h. In this case, the antioxidant mechanisms, activated by R1 and R2, were able to contrast ROS development. It has already been demonstrated that betalains work as strong anti-oxidant agents through a direct scavenger mechanism exerted by hydroxyl and imino groups and through the regulation of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression and the increase of phase II enzymes levels (Tesoriere et al., 2012; Esatbeyoglu, et al., 2015). As ROS development was abolished in CaCo-2 cells that had taken R1 and R2 24 hours before H_2O_2 treatment, it could be hypothesized that Nrf2 and Phase II enzymes might be involved in the antioxidant activity of betalains. These findings support the hypothesis that R1 and R2 present in BVr belong to the family of antioxidant compounds found in foods (like vitamin E, genistein, epigallo-catechin-3-gallate, curcumin, piperine and sulforaphane) that are thought to inhibit cancer development (Su, Z.Y. et al.; 2012).

The present results also suggest that phytochemicals extracted from BVr might have very useful applications in tumor chemoprevention *in vitro* and *in vivo* studies: specific nutraceutical preparations or food derived mixtures, enriched with XVX and betalains from red beetroot or possibly from the

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food colorant E162 (widely used for human food preparations), might be proposed as diet components, with the aim of reducing and limiting cancer onset and development.

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Abbreviations

Bax = Bcl2-like protein 4

Bcl2 = B-cell leukemia/lymphoma 2

BLS = Betalains

BVc = *Beta vulgaris* var. *cicla* L.

BVr = *Beta vulgaris* var. *rubra* L.

COX-2 = Cyclooxygenase-2

CRC = Colorectal carcinoma

DAD = Diode array detector

DCFH-DA = 2',7'-dichlorodihydrofluorescein diacetate

ESI = electrospray ionisation

GAE = Gallic Acid Equivalent

HPLC-MS = High Pressure Liquid Cromatography-Mass Spectrometry

IL8 = Interleukin 8

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LPS = Lipopolysaccharide

MDR = Multi Drug Resistance

Nrf2 = Nuclear factor (erythroid-derived 2)-like 2

ORAC = Oxygen Radical Antioxidant Capacity

PARP1 = Poly ADP-Ribosyl Polymerase 1

PGE2 = Prostaglandin E2

ROS = Reactive Oxygen Species

SRB = Sulforhodamine B

XVX = Vitexin-2-O-xyloside

Conflicts of interest

The authors declare no conflict of interest.

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Figures captions

Figure 1. Chromatographic profile obtained from the DEAE fast flow column loaded with red beetroot extract and HPLC chromatogram of the whole beetroot extract and of the separated fractions. A) R1, R2, R3 indicate the betalains peaks, obtained by reading absorbance of the fractions at 477 nm (R1 and R3) and 536 nm (R2). R1 was eluted with a 0-0.08 M NaCl gradient in 10 mM Na-phosphate buffer pH 7.5; R2 was eluted with 0.08 M NaCl isocratic in the same buffer; R3 was eluted with a 0.08-0.5 M NaCl gradient in the same buffer. B) HPLC chromatogram of beetroot whole extract: peak 1 indicates vulgaxanthin I, peak 2 betanin, peak 3 isobetanin, peak 4 betanidin. $^{\lambda}$ It indicates the change of reading Absorbance from 477 to 536 nm at 25 min.

Figure 2. DCF-DA assay in CaCo-2 cells treated with phytochemicals before H_2O_2 treatment. **CTRL**, untreated cells; **H_2O_2**, cell treated with 1 mM H_2O_2 for 1 h; **R1**, cells treated with 0.35 µg/ml R1 for 24 h and then with 1 mM H_2O_2 for 1 h; R2, cells treated with 0.25 µg/ml R2 for 24 h and then with 1 mM H_2O_2 for 1 h; **R1 + R2**, cells treated with 0.35 µg/ml R1 + 0.25 µg/ml R2 for 24 h and then with 1 mM H_2O_2 for 1 h. Data are expressed as average percentage of positive cells. At least three fields were evaluated and 100-200 cells were counted in each experiment. $^{**}p < 0.01$.

Figure 3. Cytotoxic effects of XVX and R1 and R2 on CaCo-2 cells after 24, 48 and 72h treatments. The % of cells mortality induced by XVX alone (A), R1 or R2 alone (B) and the combinations of XVX + R1, XVX + R2 and XVX + R1 + R2 (C), compared to control untreated cells (that had a mean of 5% cells mortality), is shown. Figure 3B inset shows the hormetic effect of the cytotoxicity induced by R1 and R2 at 24h, which exhibited different cytotoxicity levels when used at low or high concentrations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not statistically significant.

Figure 4. Detection of apoptosis in XVX, R1 and R2 treated CaCo-2 cell line by Western blot analysis. Cells were not treated (CTR); or treated with XVX (25 µg/ml), R2 (0.25 µg/ml), R1 (0.35 µg/ml), XVX + R2 + R1 at the same concentrations for 24 h. Bcl2, Bax, cleaved PARP1 (PARP) and cleaved caspase 3 levels were assessed in cell lysate of CaCo-2 colon cancer cell line. The molecular

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weight (MW), expressed in kDa, of each apoptosis molecular target is shown on the right side of the figure. Densitometric data were normalised to the levels of actin and were expressed as percentage of protein expression (mean ± SD) in treated samples with respect to the control. Three replicate experiments with three samples per replicate were performed and the results were analysed. * $p<0.05$; n.s., not statistically

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