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	innovative treatment, which is effective against two types of cancer cells characterized by high resistance to conventional anticancer drugs.
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Antiproliferative activity of vitexin-2-O-xyloside and avenanthramides on CaCo-2 and HepG2 cancer cells occurs through apoptosis induction and reduction of pro-survival mechanisms

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Abstract

Purpose CaCo-2 colon cancer cells and HepG2 liver cancer cells represent two malignant cell lines, which show a high resistance to apoptosis induced by conventional anticancer drugs. Vitexin-2-O-xyloside (XVX) and avenanthramides (AVNs) are naturally occurring dietary agents from *Beta vulgaris* var. *cicla* L. and *Avena sativa* L., respectively. The aim of this work was to evaluate the antiproliferative effects and the reduction of the pro-survival mechanisms exerted by XVX and AVNs, used individually and in combination, in CaCo-2 and HepG2 cancer cells.

Methods XVX and AVNs were isolated by liquid chromatography and characterized by HPLC-PDA-MS. The XVX and AVN antiproliferative effects were evaluated through sulforhodamine B method, while their pro-apoptotic effects through caspase activity assays. RTqPCR was used to investigate the modulation of the pro-survival factors Baculoviral Inhibitor of apoptosis Repeat-Containing 5 (BIRC5), Hypoxia Inducible Factor 1A (HIF1A) and Vascular Endothelial Growth Factor (VEGFA). Cellular antioxidant activity (CAA) was investigated by means of DCFH-DA assay, whereas chemical antioxidant capacity was evaluated by the ORAC method.

Results XVX and AVNs, both individually and in combination, inhibited the proliferation of CaCo-2 and HepG2 cancer cells, through activation of caspases 9, 8 and 3. XVX and AVNs downregulated the pro-survival genes BIRC5, HIF1A and VEGFA. The CAA assay showed that AVNs exhibited strong antioxidant activity inside both CaCo-2 and HepG2 cells.

Conclusions The antiproliferative activity of the XVX+AVNs mixture represents an innovative treatment, which is effective against two types of cancer cells characterized by high resistance to conventional anticancer drugs.

Keywords Apoptosis; avenanthramides; CaCo-2 colon cancer cells; cellular antioxidant activity; HepG2 liver cancer cells; vitexin-2-O-xyloside.

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Introduction

Beta vulgaris var. *cicla* L. (BVc; Chenopodiaceae) contains flavonoids that derive from apigenin, namely vitexin, vitexin-2-O-rhamnoside and vitexin-2-O-xyloside (XVX), which can exploit antiproliferative activity in several tumor cell lines [1, 2]. Studies have revealed that seeds of BVc are particularly rich in XVX, which has been purified in our laboratory and tested against RKO cells [3]. In these cells, it has been shown that XVX was able to induce apoptosis and to block the cell cycle in the G1 phase [3]. Moreover, in CaCo-2 and LoVo cancer cell lines, it has been shown that XVX was able to synergize with other anticancer phytochemicals, without any significant effect on the proliferation rate of normal cell lines [4, 5].

Oats (*Avena sativa* L.), which are normally consumed as whole grains, contain several antioxidant compounds, such as tocols, flavonoids, phenolic acids and avenanthramides [6]. Avenanthramides (AVNs), a group of substituted N-cinnamoylanthranilic acids, are soluble polyphenols exclusively found in oats, among cereals. More than 20 forms were identified by Collins in 1989, but the three most abundant are: AVN A, B and C, containing the *p*-coumaric, ferulic and caffeic acids, respectively, in the cinnamic moiety and the 5-hydroxyanthranilic acid in the anthranilic moiety [7].

AVN concentration varies considerably among varieties and it is influenced by genetic and environmental factors [8], as well as by the germination process [9, 10].

The bioavailability of AVNs has already been demonstrated in hamsters, rats and humans [11-13]. AVNs exhibit antioxidant, anti-inflammatory and anti-irritant activities *in vitro* and *in vivo* [14] and anti-proliferative properties, *in vitro* [15].

Apoptosis is a specific mechanism of cell death, which regulates tissue homeostasis, through the elimination of potentially deleterious cells [16]. Apoptosis is controlled by two pathways: the intrinsic pathway is regulated by the initiator caspase 9, leading to activation of executioner caspase 3; the extrinsic pathway is triggered by the interaction of the death ligand with its death receptor, leading to activation of initiator caspase 8 and then of caspase 3 [17].

The apoptotic program is controlled by a family of proteins called Inhibitor of Apoptosis Proteins (IAPs). Survivin, codified by the gene BIRC5, is a member of the IAPs family, that acts as a suppressor of apoptosis, through the inhibition of cleavage and activation of caspase 9 [18]. Survivin is over-expressed in several human neoplasms and it is currently used as a prognostic marker [19].

Other important factors, which control the survival of over-proliferating tumor cells, are the genes involved in angiogenesis and tumor progression [20]. These genes are mostly regulated by hypoxia inducible factors (HIFs) [21, 22]. HIFs are heterodimers of two subunits: the constitutively expressed HIF1β and the oxygen responsive HIF1α; the latter is codified by the gene HIF1A [23]. HIF1A plays an oncogenic role in colorectal cancer, since its overexpression has been associated with higher mortality rate and shorter survival [24].

The protein HIF1α controls the expression of several genes, such as VEGFA, which encodes for Vascular Endothelial Growth Factor (VEGF), the principal regulator of angiogenesis during tumor growth [25, 26]. VEGF is also able to inhibit apoptosis through up-regulation of IAPs [27].

CaCo-2 colon cancer cells are characterized by a mutated TP53 gene and overexpression of anti-apoptotic factors, which confer resistance to anticancer drugs [28]. HepG2 liver cancer cells are characterized by a wild type TP53 gene and mutations in the tumor suppressor gene Cyclin-Dependent Kinase inhibitor 2A [29], which confer high resistance to apoptosis induction [30]. From a pathophysiological point of view, colorectal carcinoma and hepatocarcinoma are linked, because the colorectal cancer is able to invade the primary tissue and generate liver metastases [31]. Therefore a drug, which shows remarkable activity against both cancer types, is relevant for chemoprevention.

This study was undertaken to set up the purification method of AVNs from oat sprouts, in order to test the antiproliferative effect of the AVNs+XVX combination on both CaCo-2 and HepG2 cancer cell lines. The mechanisms by which these phytochemicals induce apoptosis and downregulate the cancer cell pro-survival mechanisms were investigated as well.

Materials and methods

Reagents and chemicals

Ethanol and ethyl-acetate (analytical grade) were purchased from VWR International (Radnor, USA). DMEM culture medium, fetal bovine serum (FBS), antibiotics, trypsin, MEM non-essential amino acid solution, Sulforhodamine B sodium salt, water (LC-MS grade), acetonitrile (LC-MS grade), formic acid (LC-MS grade), glacial acetic acid, 2,2'-Azobis (2-amidinopropane) dihydrocloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid (Trolox), Fluorescein, H_2O_2, $HBSS^{--}$ (without calcium and magnesium) and DCFH-DA were purchased from Sigma-Aldrich (St. Louis, USA).

AVN purification

Oat sprouting

Oat grains, provided by the local producer Terra Bio Soc. Coop. (Urbino, Italy), were steeped and germinated for 14 and 72 hours, respectively, using an automatic sprouter (Fresh Life 2000, Corrupad Korea Co. Ltd., Korea), following the methods previously reported [9, 10]. Steeped and germinated oat grains were freeze-dried and stored at -20° C until analysis. Samples were milled in a ZM 200 ultracentrifugal mill with a 0.5 ring sieve (Retsch, Haan, Germany).

Extraction and isolation of AVNs

AVNs were extracted from milled oat with acidified ethanol (1:10 w/v), following the procedure reported by us [8], with the exception that ethanol was acidified with 0.1% glacial acetic acid, instead of 10 mM H_3PO_4 buffer.

The raw extract was concentrated by rotary evaporation to 1/10 of the initial volume. To remove lipophilic components from the extract, we followed the procedure reported by Liu et al [32], with some modifications. Briefly, Octyl Sepharose™ CL 4-B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added to the extract (0.5 ml per g extracted). The mixture was completely dried, re-suspended in acidified 50% ethanol and transferred to a glass chromatography column containing Octyl Sepharose™ CL 4-B, previously pre-equilibrated in acidified 50% ethanol. The column was then eluted with 3 x bed volume (V_b) of acidified 50% ethanol. The eluate was concentrated under vacuum, at 40°C, by rotary evaporation.

The defatted dried extract was dissolved in acidified 30% ethanol and purified on a Sephadex LH-20 column (50 x 150 mm; Sigma-Aldrich Inc., St. Louis, USA), equilibrated in acidified 30% ethanol, and controlled by the LC-Chromatograph AKTA Purifier 10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mobile phase was changed as follows: 2 x Vb of acidified 30% ethanol in isocratic mode to remove amino acids, free sugars, proteins, etc. [32]; 2 x Vb of acidified 50% ethanol in isocratic mode to remove the flavonoids and phenolic acids [32]; 2 x Vb of acidified ethanol from 50 to 95% in gradient mode, to recover the absorbed AVNs. The flow rate was 5 ml/min and absorbance values were obtained at 330 nm.

HPLC-PDA-MS analysis of AVNs

The dried fractions obtained from the Sephadex LH-20 column chromatography were dissolved in ethanol and directly analyzed in a Water instrument equipped with Alliance HT 2795 High Performance liquid Chromatography (HPLC), 2996 Photo Diode Array (PDA) and Micromass LC/MS ZQ 2000 detector, following the procedure reported by us [8].

To identify the individual AVNs in the HPLC chromatograms, retention time, UV spectra, MS ESI (+) and ESI (-) spectra were compared with those of commercially available external standards (AVN A, B, C, Sigma-Aldrich, St. Louis, USA), and the peak areas were used for quantification.

XVX purification

XVX was isolated from seeds of BVc, obtained from Suba Seeds Company S.p.A. (Longiano, Italy). The extract from seeds was prepared by solid-liquid extraction with 70% ethanol in a Timatic extractor (Tecnolab S.r.l., Spello, Italy) and liquid-liquid extraction with ethyl-acetate. Two chromatography columns were applied: a Diaion HP-20 (Sigma-Aldrich, St. Louis, USA) and a Davisil C18 (ALLTECH, Milan, Italy), performed by means of the LC-Chromatograph AKTA Purifier 10, following the elution of XVX at 336 nm [3].

The HPLC analysis of XVX was performed using the same HPLC equipment used for AVNs, but a spherisorb ODS2 column (4.6 x 250 mm; Waters Corporation, Milford, USA), with 5 µm particle size was used. Standard and sample injected volumes were 20 µl. The gradient consisted of 0.01% acetic acid in water (solvent A) and methanol containing

0.01% acetic acid (solvent B), developed as follows: from 0 to 60% solvent B in the interval 0-45 min at 1 ml/min, then to 100% B in the interval 45-50 min. XVX was identified using a purified standard available in our lab [3].

ORAC Assay

Antioxidant activity was assessed using the ORAC (Oxygen Radical Absorbance Capacity) assay and a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany) equipped with a temperature-controlled incubation chamber and automatic injection pump according to Ninfali et al [33]. Fluorescence was read at 485 nm ex. and 520 nm em. until complete extinction. ORAC values were expressed as µmol Trolox Equivalents (TE) g^{-1} d.m, which was used as external standard.

Cell cultures

HepG2 liver cancer cell line and CaCo-2 colon cancer cell line were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µM MEM non-essential amino acid solution. NCTC 2544 normal human keratinocytes were obtained from Interlab Cell Line Collection (ICLC, Genova, Italy) and maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Sulforhodamine B (SRB) assay

Cells (10^4/well for CaCo-2 and NCTC 2544; $2x10^4$/well for HepG2) were plated in 96-well plates and treated with XVX and AVNs, both individually and in combination, for 24, 48 and 72 hours. At the end of treatment, cells were incubated with SRB as previously reported [5]. The results were expressed as the percentage of cell viability, compared to control untreated cells (100% viability).

DCFH-DA assay for Cellular Antioxidant Capacity (CAA) evaluation

Cells were seeded ($2x10^5$ for CaCo-2 and $3x10^5$ for HepG2) in 6-well plates and treated for 24h with P4 or P3. After removing of cell medium, the production of ROS in CaCo-2 and HepG2 cells was monitored following the procedure reported by Farabegoli et al [5], with the substitution of PBS with HBSS $^{--}$ only. After incubation, the cells were observed at microscope with a green FITC filter (OLYMPUS IX51). Cells showing a bright and intense green fluorescence were counted as positive, whereas cells with no or low fluorescence were counted as negative. Three fields per sample were analyzed and each time at least 100 cells were counted. Three independent experiments were performed for both CaCo-2 and HepG2 cells and results were expressed as % DCF fluorescent positive cells versus control. The images were obtained using the software OLYMPUS Ps-blm1.

Determination of caspase 3, caspase 9 and caspase 8 activities

HepG2 and CaCo-2 cells were untreated, or treated with 50 µM XVX, 120 µM P3, 120 µM P4 or with 50 µM XVX+120 µM P4 for 48h, then the Petri dishes were put in ice and the cytosols were extracted following the procedure previously reported [34].

Caspase 3, caspase 9 and caspase 8 activities were assessed through the Caspase Colorimetric Assay Kit (BioVision, Milpitas, USA) according to the manufacturer's instructions. 100 µg of cytosol from untreated or phytochemicals treated HepG2 and CaCo-2 cells were incubated with caspase 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) DMQD-pNA specific for caspase 3, or LEHD-pNA specific for caspase 9, or IETD-pNA specific for caspase 8 was added and the samples were transferred in a 96-wells microplate. All samples were incubated at 37ºC for 2h. The absorbance, representing the activities of caspases, was measured at λ=405 nm in a microplate reader (BioRad Laboratories, Hercules, USA).

RNA extraction, cDNA production and RTqPCR experiments

Total RNA was extracted from CaCo-2 and HepG2 cells using the TriReagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. cDNA production and RTqPCR assays were performed as previously reported [34]. The following list of primers was used: β -Actin F: 5'-GCGAGAAGATGACCCAGATC-3' , R: 5'-GGATAGCACAGCCTGGATAG-3' (77bp), BIRC5 F: 5'-ATTCGTCCGGTTGCGCTTTCC-3', R: 5'-CACGGCGCACTTTCTCCGCAG-3'(162bp), HIF1A F: 5'-TCTGGGTTGAAACTCAAGCAACTG-3', R: 5'-CAACCGGTTTAAGGACACATTCTG-3' (150 bp), VEGFA F: 5'-TCACAGGTACAGGGATGAGGACAC-3', R: 5'-CAAAGCACAGCAATGTCCTGAAG-3' (72 bp).

Statistical analysis

Data were expressed as mean ± SD. Statistical significance was assessed by one-way ANOVA, using PRISM 5.1 (GraphPad Software, La Jolla, USA). The results were considered statistically significant when $p < 0.05$.

Results

Purification of AVNs

AVNs were purified from oat sprouts, using the Sephadex LH-20 column chromatography. We obtained four main peaks, labeled as P1-P4 (Fig. 1a). The HPLC-PDA-MS analysis revealed that P1 and P2 peaks, contained hydrophilic contaminants and were discarded; P3 and P4, both containing AVNs, were collected and concentrated to dryness. From the HPLC-PDA-MS analysis, P3 revealed the presence of AVN B only, at 95% purity level (Fig. 1b); P4 revealed the presence of a mixture of AVN A, B, C, representing 80% of the whole weight (Fig. 1c). The structural formulae are shown in Fig. 1b and 1c. The relative % ratios of the AVN A, B, C of P4 are reported in Table 1. The concentrations

were accurately evaluated by HPLC, referring to calibration curves made with commercial AVN A, B, C standards. The remaining 20% of the P4 fraction was characterized by minor AVN forms (Fig. 1c; peaks 1-4), which were quantified using the commercial AVN A as external standard [32]. Our protocol for the chromatographic purification of AVN forms gave a reproducible profile in several repeated experiments (n=3). Our profile slightly differs from those of other authors [32]. It is worth to remember here that in the presentation of the following results, we used the label P3 for AVN B and P4 for the AVN mixture (AVN A, B, C).

Antioxidant capacity evaluation

The antioxidant capacity, evaluated through the ORAC method, showed ORAC values of 6,547 ± 290 and 19,079 ± 474 µmol TE/g for P3 and P4, respectively (Table 1). The higher ORAC value of the P4 fraction should be related to the presence of AVN C, which is considered the most potent antioxidant form [35].

Purification of XVX

XVX was purified in our laboratory from BVc seeds at 95% purity level. The XVX powder, stored at -20C, was used alone or in combination with AVNs for cell treatments. The purification protocol was tested along several years of reproducible experiments [3].

Antiproliferative effects of individual phytochemicals

Earlier studies on normal human cells allowed us to choose the concentration range of XVX, P3 and P4 to be used in the dose response experiments of antiproliferative activity. As no cytotoxic effects were detected by us when XVX was used in the range 0-100 µM and when AVNs were used in the range 0-120 µM on normal NCTC 2544 cells (Online resource 1), we planned to evaluate antiproliferative effects on CaCo-2 and HepG2 cancer cells in this concentration range for XVX and for P3 and P4.

Fig. 2 shows the antiproliferative effects on CaCo-2 colon cancer cells (a, c, e) and HepG2 liver cancer cells (b, d, f) of the individual phytochemicals at 48h. At this treatment time, the maximum antiproliferative effect occurred.

On CaCo-2 cells, the IC_{50} values, at 48h were: 50.9 ± 5.5 µM, 126.5 ± 12.5 µM, 114.6 ± 5.5 µM, for XVX, P3 and P4 respectively. Otherwise, on HepG2 cells, the IC_{50} values, at 48h were: 71.6 ± 7.4µM, 182.7 ± 18.1 µM, 39.9 ± 4.1 µM for XVX, P3 and P4 respectively. Therefore, the XVX treatment was the most cytotoxic on CaCo-2 cells, while the P4 treatment was the most cytotoxic on HepG2 cells.

Modulation of the caspase activities by individual phytochemicals

XVX, P3 and P4 were used to investigate, at 48h, the ability of these phytochemicals to activate both the initiator caspases 8 and 9 and the effector caspase 3 in CaCo-2 and HepG2 cancer cells (Fig. 3).

The caspase 3 activity significantly increased on both CaCo-2 (Fig. 3a) and HepG2 (Fig. 3b) cancer cells with P4 and XVX treatments; otherwise, P3 was effective in CaCo-2 cells only.

The caspase 9 activity, linked to the "intrinsic" pathway of apoptosis, increased in the presence of XVX, but not in the presence of P4 or P3, on both CaCo-2 (Fig. 3c) and HepG2 (Fig. 3d) cancer cell lines.

In CaCo-2 cells, the caspase 8 activity, linked to the "extrinsic" apoptotic pathway, significantly increased in the presence of P4 and P3, but not in the presence of XVX (Fig. 3e); otherwise in HepG2 cells, only P4 was able to induce a significant increase in caspase 8 activity levels (Fig. 3f).

In other words, XVX activates the intrinsic apoptotic pathway, while AVNs activate the extrinsic apoptotic pathway, in both colon and liver cancer cells (Fig. 3).

Cellular antioxidant activity (CAA) of P4 and P3

We incubated the CaCo-2 and HepG2 cells with P3 and P4, individually, in order to test their CAA, which is also an indication of the permeability of the phytochemicals through the cell membranes. The CaCo-2 cells (Fig. 4a) were treated for 24h with 60 µM P4 and 60 µM P3, whereas the HepG2 cells (Fig. 4b) with 30 µM P4 and 120 µM P3, before generating ROS by 1 mM H_2O_2 for 1h. The choice of P4 and P3 concentrations was based on the minimum phytochemical concentration that showed a statistically significant antiproliferative effect on CaCo-2 and HepG2 cancer cells (Fig. 2).

P4 exerted a stronger CAA than P3 in both CaCo-2 (Fig. 4c) and HepG2 (Fig. 4d) cells, remarkably reducing the number of green fluorescent cells, after treatment with H_2O_2. These results suggest a link between the AVNs CAA and their antiproliferative effects on CaCo-2 and HepG2 cancer cells.

The intracellular antioxidant capacity of XVX was not investigated here, as it has already been demonstrated elsewhere [4, 36].

Molecular effects of the XVX+P4 mixture

P4 only was chosen to be tested for the antiproliferative activity in combination with XVX. This choice was due to its lower IC_{50} value, to the greater induction in caspase 8 and caspase 3 activity levels and to the higher CAA exerted in both cancer cell lines, when compared to P3.

CaCo-2 and HepG2 cells were incubated for 48h with XVX maintained at 50 µM and P4 at the increasing concentrations of 30, 60 or 120 µM. The combination 50 µM XVX+120 µM P4 showed the greatest antiproliferative effects on both cancer cell lines (Fig. 5a, 5b) and induced also the highest increase in the activity levels of caspase 3, 9 and 8 in both CaCo-2 and HepG2 cancer cell lines (Table 2).

XVX+P4 mediated downregulation of BIRC5, HIF1A and VEGFA expression levels

Through RTqPCR assays, we assessed whether XVX and P4, used alone and in combination, were able to reduce the expression levels of the pro-survival genes BIRC5, HIF1A and VEGFA in CaCo-2 and HepG2 cancer cells.

Our results (Fig. 6) showed a downregulation of the expression levels of the anti-apoptotic gene BIRC5 (survivin) by XVX and XVX+P4 on CaCo-2 (Fig. 6a) and HepG2 (Fig. 6b) cells. The treatment with P4 alone was ineffective on both cancer cell lines (Fig. 6a, 6b).

Individually, XVX and P4 were able to significantly downregulate the expression levels of HIF1A on both CaCo-2 (Fig. 6c) and HepG2 (Fig. 6d) cancer cells. In addition, XVX+P4 showed a remarkable reduction ($p<0.001$) in HIF1A expression levels in both cancer cell lines (Fig. 6c, 6d).

XVX and P4, used alone, were also able to decrease the expression levels of VEGFA on both CaCo-2 (Fig. 6e) and HepG2 (Fig. 6f) cancer cells. The combination XVX+P4 showed a remarkable downregulation of the expression levels of VEGFA, with a greater extent on HepG2 than on CaCo-2 cells (Fig. 6e and 6f). In synthesis, XVX and P4 (AVNs), much better in combination than alone, markedly downregulated the expression levels of the genes HIF1A and VEGFA on both cancer cell lines, confirming data obtained with other flavonoids [37].

Discussion

The phytochemicals XVX and AVNs are purified in our lab from *Beta vulgaris* var *cicla* L. and *Avena sativa* L., respectively. Green beet and oat are foods widely consumed in the Western and Eastern countries. With the ingestion of these foods, the above nutrients are absorbed in the gut and reach the liver through entero-hepatic recirculation [38]. By this way, these phytochemicals are bioavailable and able to exploit their biological effects in both colon and liver cells [38].

When a tumor develops in the colon, the cancer cells may spread into the liver and metastatize there [31]. Therefore, a couple of phytochemicals able to exploit biological effects on both colon and liver tissues, represent an important chemopreventive tool for subjects exposed to colon cancer risks, such as hereditary adenomatous polyposis [31].

In our study, we have evaluated the antiproliferative effects of a combination of XVX and AVNs, in an *in vitro* model of colon and liver cancer cell lines. Our results showed that XVX and AVNs, used individually, were able to inhibit the proliferation of both CaCo-2 and HepG2 cancer cells, without inducing cytotoxic effects on normal human NCTC 2544 cells. Regarding the two AVN fractions that we isolated, P4 (AVNs mix) was more active as an antiproliferative agent, than P3 (AVN B). This may be explained by a synergy among the AVN A, B, C forms contained in P4, an hypothesis which is sustained by the higher ORAC and CAA of P4, compared to P3.

Our results also demonstrated that the combination XVX+AVNs mix (P4) showed a greater inhibition of proliferation rate than XVX and AVNs used alone. This antiproliferative effect was exerted through the ability of the XVX+AVNs to activate both intrinsic and extrinsic apoptotic pathways. In fact, caspase 3 activity was increased to a greater extent by XVX+AVNs than by the individual compounds, in both cancer cell lines. It has already been shown that XVX exerts its

anticancer effect through the activation of the effector caspase 3 in several cancer cell lines [5, 34], but this is the first time that such a remarkable increase in caspase 3 activity was identified in the presence of the couple XVX+AVNs in CaCo-2 and HepG2 cancer cell lines.

Another interesting aspect of this report was the effect exerted by our phytochemicals on the modulation of some genes, which are fundamental for cancer cell survival.

In fact, our data showed that the XVX+AVNs combination remarkably reduced the expression levels of the pro-survival factors: BIRC5, HIF1A and VEGFA. The rationale for the choice of these genes among the many others possible markers, in this research, derived from previous works, showing that XVX downregulated BIRC5 in T24 bladder cancer cells [34], and vitexin, the mono glucoside of apigenin, was able to decrease the activity of HIF1α and downregulate the expression levels of its target gene VEGFA [39]. Our results confirm the previous results and underline the remarkable capacity of the XVX+AVNs combination to reduce the anti-apoptotic defenses of the CaCo-2 and HepG2 cancer cell lines, influencing the expression of the members of the IAPs family, in which survivin, the BIRC5 gene product, is enclosed [40].

Notably, XVX treatment was the most cytotoxic on CaCo-2 cells, while the AVNs treatment was the most cytotoxic on HepG2 cells, but when utilized in the mixture, the antiproliferative effect of XVX+AVNs combination was greater in HepG2 than in CaCo-2 cells. We hypothesized that this different behavior could be linked to the different genetic background of the two cancer cell lines, as CaCo-2 cells possess a mutated TP53 gene, whereas HepG2 cells a wild type TP53 gene. We believe that in HepG2 cells, the p53 protein levels could be increased by the phytochemical combination, leading to activation of apoptosis, as it was demonstrated by others for all-trans retinoic acid [29]. However, the clarification of this aspect deserves further research.

In conclusion, our work shows that the combined action of XVX and AVNs (P4) induces both intrinsic and extrinsic apoptotic pathways on CaCo-2 and HepG2 cancer cells, as well as by the downregulation of the pro-survival factors BIRC5, HIF1A and VEGFA in both cancer cell lines.

XVX and AVNs derive from renewable vegetable sources and their purification can be scaled up for a wider request of these phytochemicals for nutraceutical preparations, as the synthesis of these molecules is difficult, expensive and, for XVX, not fully feasible. Our *in vitro* study creates the basis for the possible use of this combined treatment for chemoprevention against the insurgence of both colon and liver cancers also *in vivo*.

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Abbreviations

AVNs, avenanthramides

BIRC5, Baculoviral Inhibitor of apoptosis Repeat-Containing 5

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

CAA, Cellular Antioxidant Activity

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

HIF1A, Hypoxia Inducible Factor 1A

HPLC, High Pressure Liquid Chromatography

IAPs, Inhibitor of Apoptosis Proteins

MS, Mass Spectrometry

ORAC, Oxygen Radical Absorbance Capacity

PDA Photo Diode Array

SRB, Sulforhodamine B

UV, Ultraviolet

VEGF, Vascular Endothelial Growth Factor

XVX, vitexin-2-O-xyloside

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Figure captions

Fig. 1 Elution profile showing the four main peaks (P1-P4) obtained from the Sephadex LH-20 column chromatography loaded with the extract of milled oat (a); HPLC profile of P3 (b) and P4 (c), used for the cytotoxicity assays. P3 contains the AVN B (B); P4 contains a mixture of peaks, corresponding to AVN C (C), AVN A (A), AVN B (B) and other minor AVNs (peaks 1-4). The structural formulae are also shown

Fig. 2 The antiproliferative effects of P3, P4 and XVX on CaCo-2 colon cancer cells (a, c, e) and HepG2 liver cancer cells (b, d, f), after 48h treatment. Data were expressed as percentage of cell viability relative to the control, represented by the corresponding untreated cells. Three replicate experiments with three samples analyzed for each replicate (n=9) were performed *$p<0.05$, **$p<0.01$, ***$p<0.001$

Fig. 3 Evaluation of caspase 3, caspase 9 and caspase 8 activity levels, induced by XVX, P3 and P4 in CaCo-2 (a, c, e, respectively) and HepG2 (b, d, f, respectively) cancer cells. Cells were untreated (CTRL), or treated for 48h with P3 (120 µM), or P4 (120 µM) or XVX (50 µM). Data were expressed as % caspase 3 (a, b), % caspase 9 (c, d), % caspase 8 (e, f) activity levels. Three replicate experiments with two samples analyzed for each replicate (n=6) were performed. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Fig. 4 Representative images of DCFH-DA assay in CaCo-2 (a) and in HepG2 (b) cells treated with phytochemicals before H_2O_2 treatment. CTRL, untreated cells; H_2O_2, cells treated with 1 mM H_2O_2 for 1 h; P4 (60 µM), cells treated with 60 µM P4 for 24h and then with 1 mM H_2O_2 for 1 h; P3 (60 µM), cells treated with 60 µM P3 for 24h and then with 1 mM H_2O_2 for 1 h; P4 (30 µM), cells treated with 30 µM P4 for 24h and then with 1 mM H_2O_2 for 1 h; P3 (120 µM), cells treated with 120 µM P3 for 24h and then with 1 mM H_2O_2 for 1 h (c, d). Three fields per sample were analyzed and each time at least 100 cells were counted; three independent experiments were performed for both CaCo-2 and HepG2 cancer cells (n=9) and results were expressed as % DCF fluorescent positive cells versus control; **$p<0.01$, ***$p<0.001$

Fig. 5 The antiproliferative effect of XVX+P4 combination on CaCo-2 colon cancer cells (a) and HepG2 liver cancer cells (b), after 48h treatment. Data were expressed as percentage of cell viability relative to the control, represented by the corresponding untreated cells. Three replicate experiments with three samples analyzed for each replicate (n=9) were performed **$p<0.01$, ***$p<0.001$

Fig. 6 Evaluation, through RTqPCR assay, of the effects of XVX, P4 and their combination on BIRC5, HIF1A and VEGFA expression levels in CaCo-2 (a, c, e) and HepG2 (b, d, f) cancer cells. BIRC5 (a, b), HIF1A (c,

d), VEGFA (e, f) mRNA levels were measured and normalized to the mRNA levels of the housekeeping gene β-actin. Results obtained from untreated cells (CTRL) and from CaCo-2 and HepG2 cells treated with XVX (50 µM), P4 (120 µM), or XVX+P4 (50 µM XVX+120 µM P4) for 24h, are shown. Three replicate experiments with three samples analyzed for each replicate (n=9) were performed. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 1

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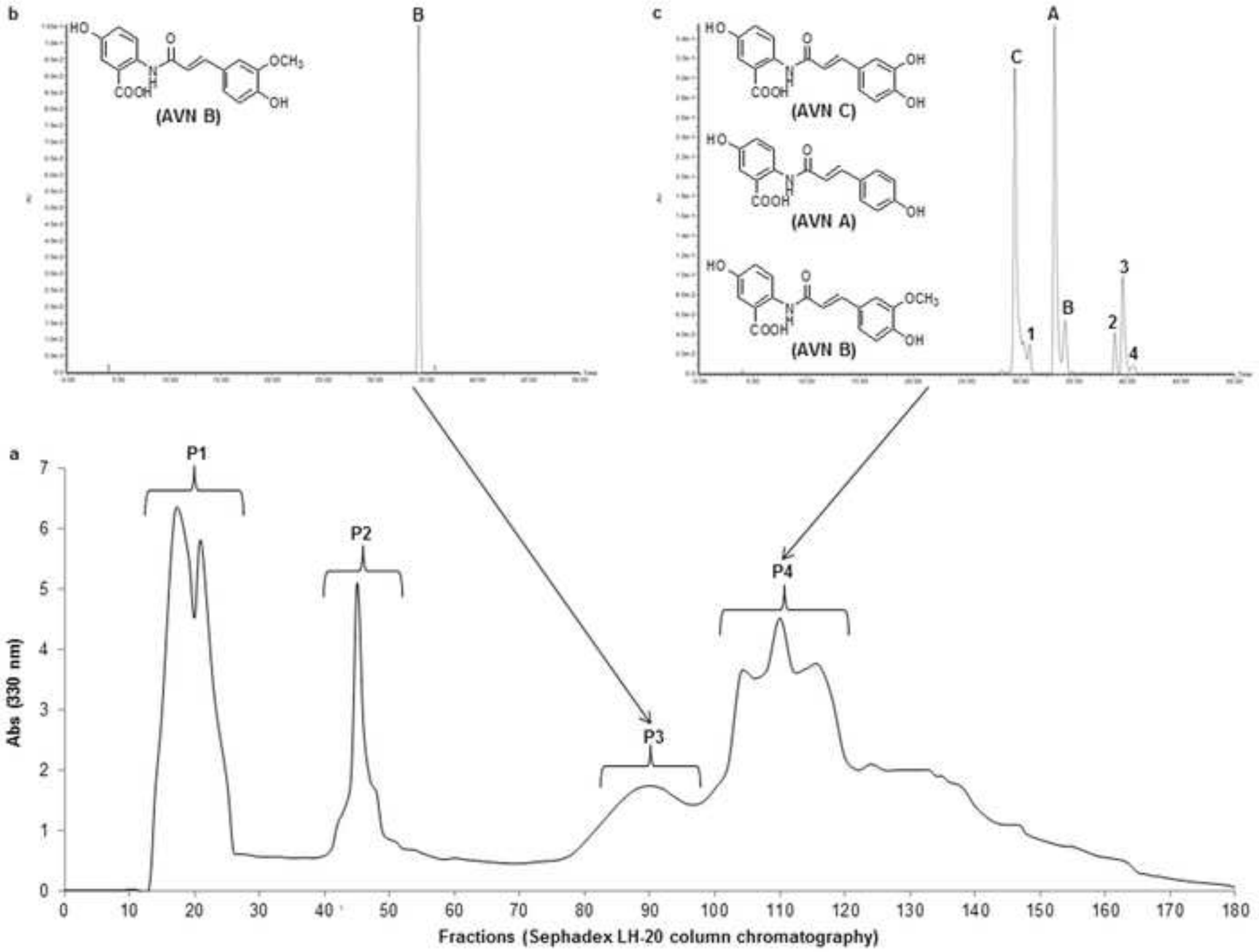

Figure 2

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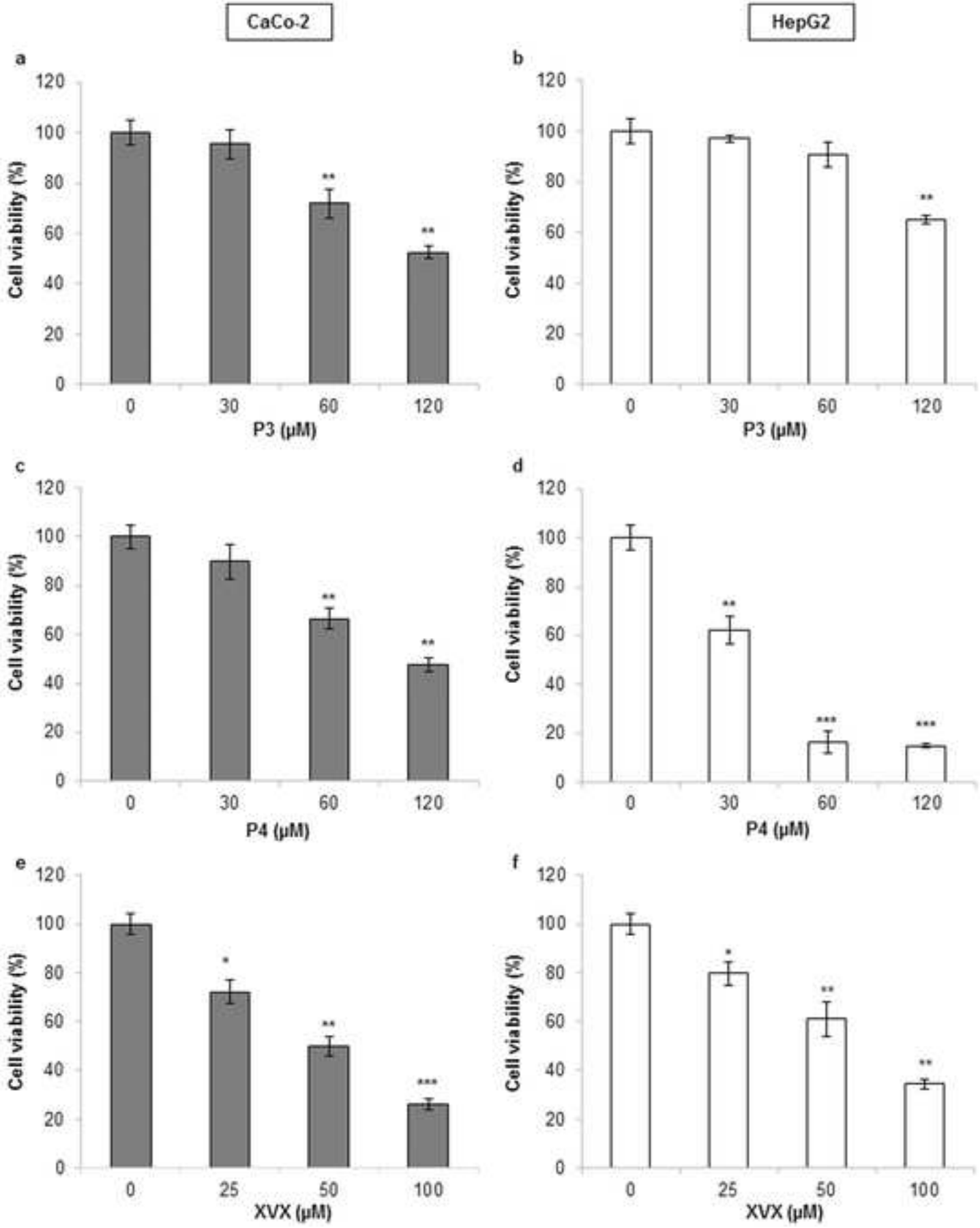

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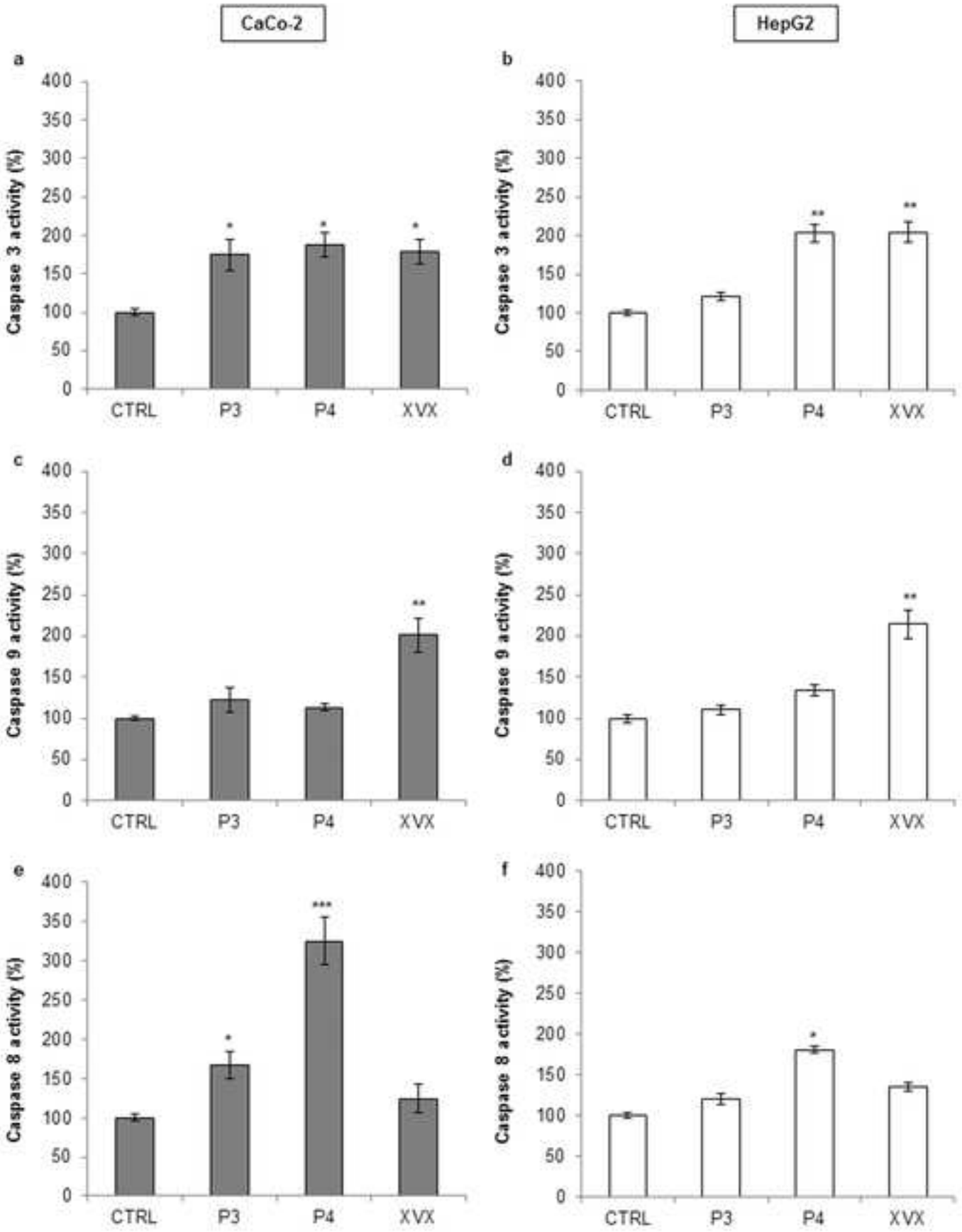

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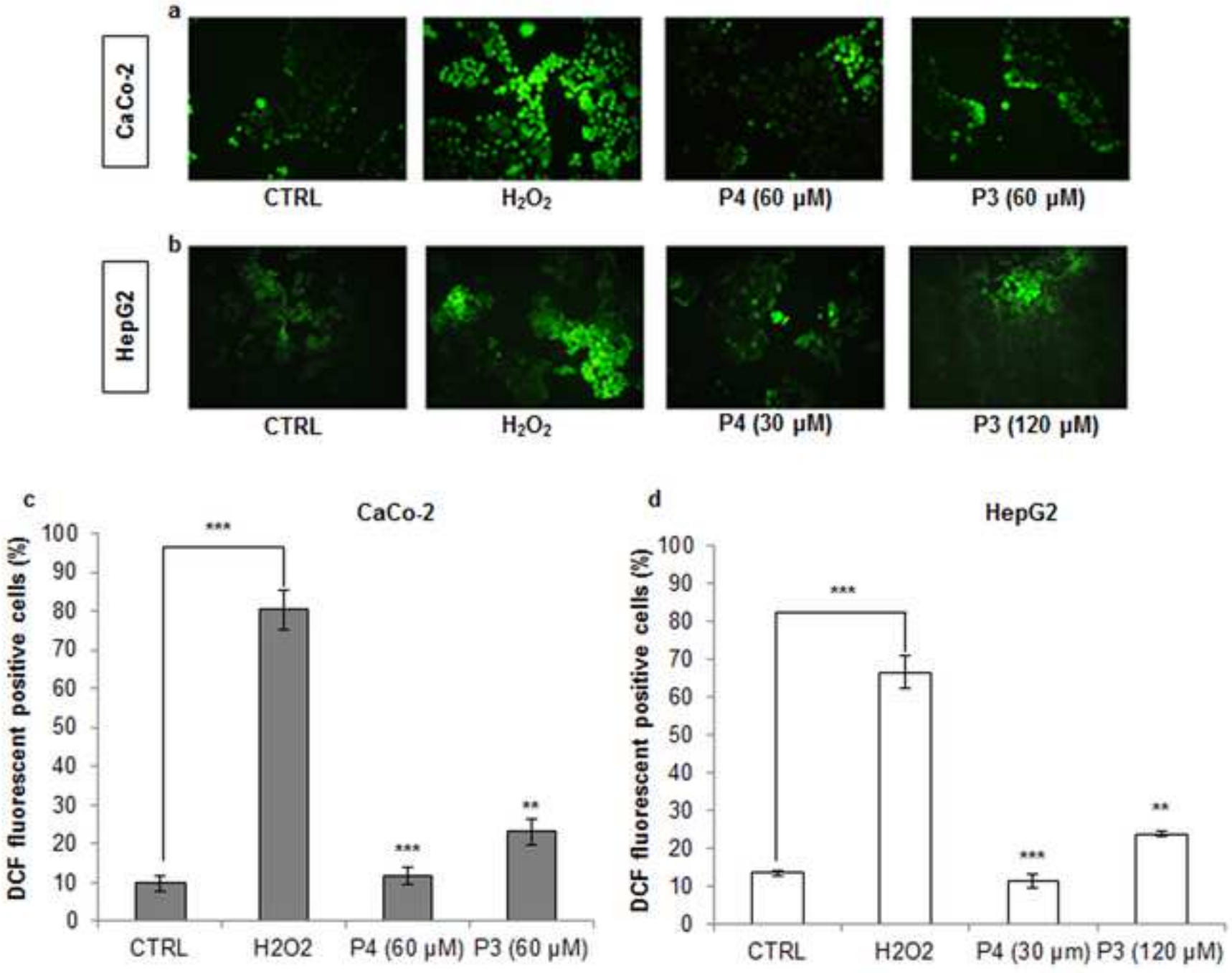

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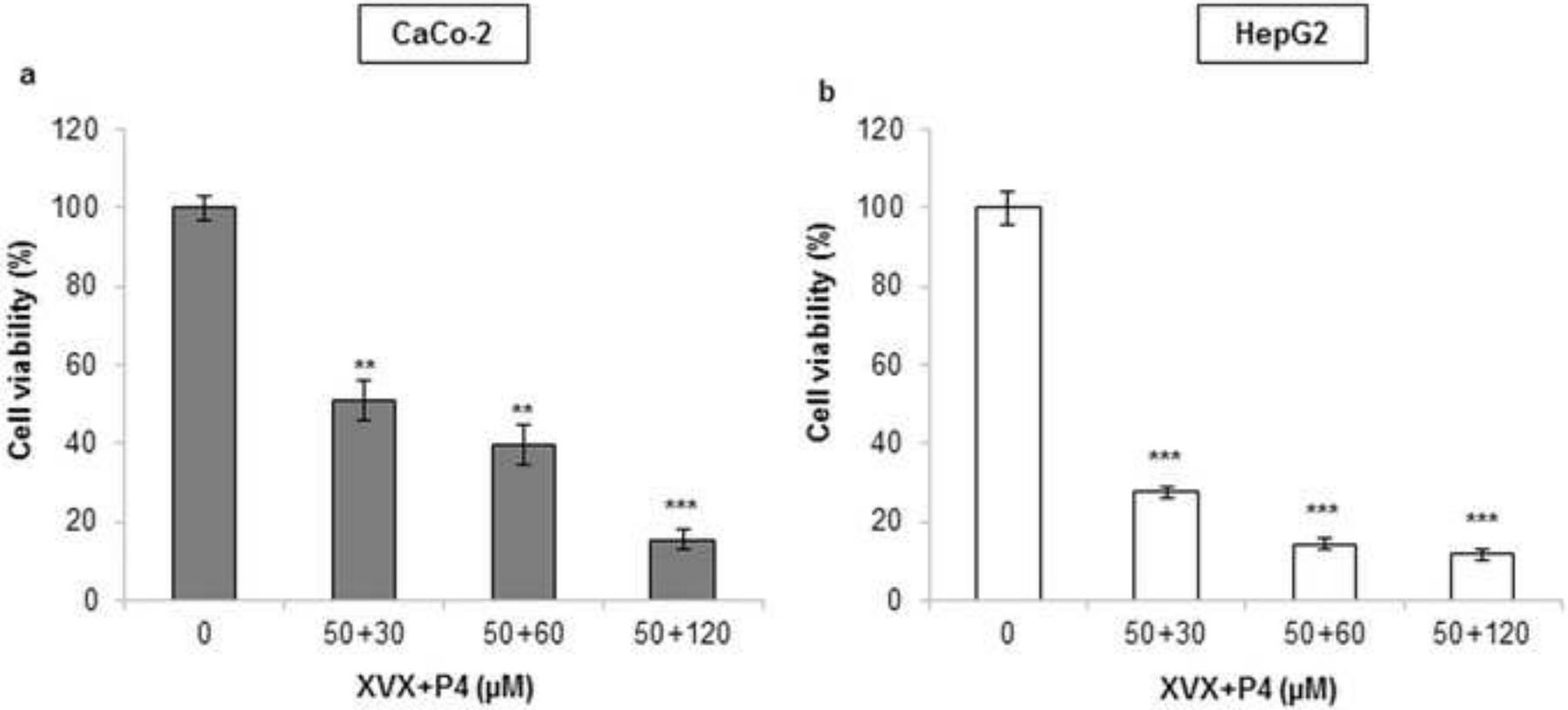

Figure 6

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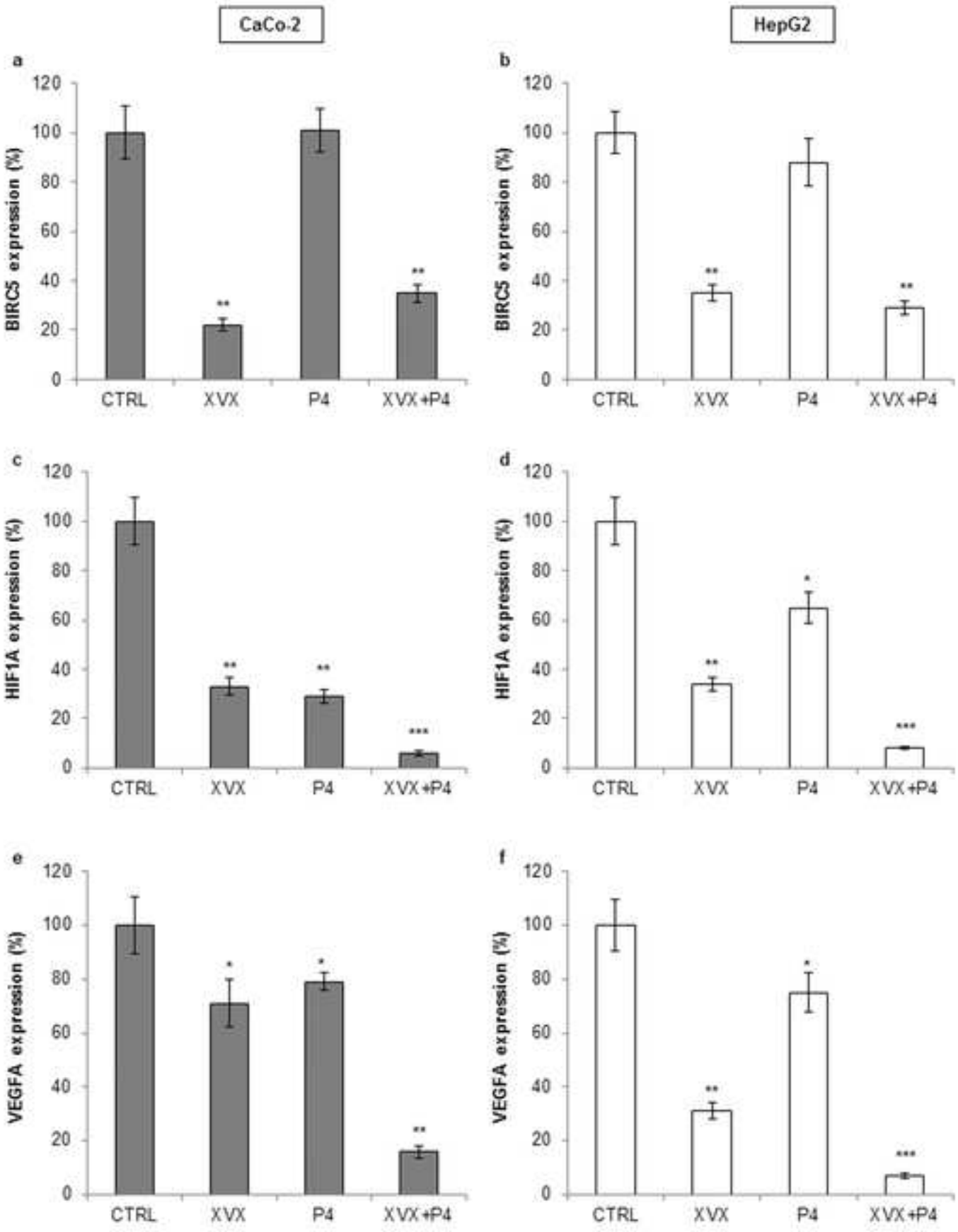

Table 1

Table 1. HPLC-PDA-MS characterization of AVNs purified from P3 and P4 fractions and antioxidant capacity (ORAC) of the dried fractions.

	AVN type	t_R[a]	%[b]	m/z[a]	UV[a] (λ_{max})	ORAC (µmolTE/g)
P3	B	34.2	95 ± 2	329	342	6,547 ± 290
	C	29.4	37 ± 2	315	343	
P4	A	33.1	35 ± 1	299	319	19,079 ± 474
	B	34.2	8 ± 1	329	342	

[a] t_R, m/z, UV spectra were compared with those of the external AVN A, B, C standards, and the peak areas were used for quantification.
[b] AVN average percentages found in the P3 and P4 fractions, obtained from three different purification batches.

Table 2. Caspases 3, 9 and 8 activity levels, after 48h treatment with XVX+P4 combination, on CaCo-2 and HepG2 cells.

	CaCo-2		HepG2	
	CTRL	**XVX + P4**	**CTRL**	**XVX+P4**
Caspase 3	100 ± 5	229 ± 17**	100 ± 4	352 ± 47***
Caspase 9	100 ± 3	205 ± 14**	100 ± 5	229 ± 26**
Caspase 8	100 ± 4	309 ± 16***	100 ± 3	191 ± 21*

Three replicate experiments with two samples analyzed for each replicate (n=6) were performed. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Cells were treated with 50 µM XVX+120 µM P4.

Online resource 1