Anti-proliferative effect of main dietary phytosterols and β-cryptoxanthin alone or combined in human colon cancer Caco-2 cells through cytosolic Ca^{+2} – and oxidative stress-induced apoptosis

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ARTICLE INFO

ABSTRACT

β-cryptoxanthin (β-Cx) and phytosterols (Ps) have potential against different cancer types, including colon cancer. However, their combined action has not been reported so far. Human colon cancer Caco-2 cells were treated 24 h with β-Cx and/or main dietary Ps (β-sitosterol, campesterol and stigmasterol), alone or in combination, at concentrations compatible with physiological human serum levels. A decrease in cell viability due to apoptosis (rise in sub-G1 population and exposure of membrane phosphatidylserine) was accompanied with dephosphorylation of BAD, mitochondrial depolarization and caspase 3-dependent PARP cleavage, with intracellular Ca^{2+} influx and increase of RONS levels as initial triggers. Ps and β-Cx, alone or in combination showed anti-proliferative activity against human colon adenocarcinoma Caco-2 cells through the mitochondrial pathway of apoptosis. No additive or synergistic effects were observed. The importance of bioactivity-guided assays with mixtures of dietary bioactive compounds to determine their eventual interactions in the functional food context is demonstrated.

1. Introduction

Diet plays a significant role in health and well-being, indeed unbalanced nutrition or inadequate diet is a key risk factor for chronic age-related diseases, cancer included (Millen et al., 2005). As many as 35% of all cancers can be prevented by proper life-style decisions, among which dietary modifications may have a prominent role (Khan et al., 2014). Colorectal cancer, which is the fourth most common cancer in men and the third most

common cancer in women worldwide, has been linked with a diet low in fruits and vegetables, physical inactivity, obesity, and smoking (Center, Jemal, Smith, & Ward, 2009). Considering that prevention rather than treatment is the desired approach, dietary modification through increased phytochemical assumption, could be a good and cost-effective strategy, since more than 500 plants and related bioactive compounds have been associated with health benefits and protection from colon cancer (Jensen, Panagiotou, & Kouskoumvekaki, 2014). Most phytochemicals affect the redox status of either healthy or cancer cells, with remarkably different cell responses at a molecular level (Nair, Li, & Kong, 2007), that possibly may interfere each other. Then, dietary approaches aimed at developing functional foods for potential cancer prevention require solid knowledge not only of the effects of individual compounds, but possibly of their mixture in proportion known to exist in natural products or even industrial preparations.

β-Cryptoxanthin (β-Cx) is a major dietary pro-vitamin A carotenoid provided by citrus juices (Granado-Lorencio, Olmedilla, Blanco, & Rojas-Hidalgo, 1996). There are scarce data about its potential to modulate the growth of cancer cells (Niranjana et al., 2014). Likewise, a few *in vivo* and *in vitro* studies suggest that β-Cx might prevent colon cancer. Indeed it has been shown that β-Cx suppresses colon carcinogenesis in male F344 rats (Tanaka et al., 2000; Tanaka, Tanaka, Tanaka, & Kuno, 2012), repairs DNA oxidation damage in human colon carcinoma Caco-2 cells (Lorenzo et al., 2009), and induces apoptosis in Colo 320 and Colo 205 human colon cancer cells (Ugocsai, Varga, Molnár, Antus, & Molnár, 2005). Biological activities of phytochemicals, including β-Cx, greatly depend on bioavailability. Serum concentration of β-Cx ranges from 0.05 to 0.52 μM, and can reach 1 μM after ingestion of juices rich of β-Cx (Lian, Hu, Russell, & Wang, 2006).

Phytosterols (Ps) are C28 and C29 carbon steroid alcohols that resemble cholesterol in structure, but are found exclusively in plants. These compounds are well known for their effects on cholesterol blood levels (Wong, 2014); however, research into their potential role in mitigating cancer risk, and specifically colon cancer, has received comparably little attention (Bradford & Awad, 2010; Grattan, 2013). It has been reported that β-sitosterol, the prevalent Ps in the diet, has anti-proliferative effects in rat models of colon cancer (Awad, Tagle-Hernandez, Fink, & Mendel, 1997; Baskar et al., 2012; Baskar, Ignacimuthu, Paulraj, & Al Numair, 2010; Raitch, Cohen, Faqzzani, Sarwal, & Takahashi, 1980) and in the colon cancer cell lines HT-29, HCT116 and Colo 320, within the range of physiological plasma concentrations (4–70 μM) (Awad, Chen, Fink, & Hennessey, 1996; Awad & Fink, 2000; Choi et al., 2003; Jayaprakasha, Jadegoud, Nagana Gowda, & Patil, 2010; Montserrat-de la Paz, Fernández-Arche, Bermúdez, & García-Giménez, 2015). In other cases, inhibition of colon cancer cell growth (Caco-2 cells) has been observed with pharmacological levels of β-sitosterol and campesterol (≥100 μM) (Daly, Aherne, O'Connor, & O'Brien, 2009). However, mechanism(s) of action of Ps have not been fully elucidated and their effects appeared to be cell-specific (Baskar et al., 2010).

Due to the well-known cholesterol-lowering property of phytosterols (Ps) (Ras et al., 2013), a variety of commercial foods have been enriched with Ps. Recently, beneficial effects have been reported from the regular consumption of a fruit-skimmed milk beverage containing β-sitosterol, campesterol and stigmasterol in combination with β-Cx. β-Cx has appeared to improve the cholesterol-lowering effect of Ps when supplied simultaneously and this combination may also be beneficial in reducing the risk of osteoporosis (Granado-Lorencio et al., 2014). Taking into account the serum concentration of β-Cx and Ps after consumption of this beverage (Alemany et al., 2013; Granado-Lorencio, Donoso-Navarro, Sánchez-Siles, Blanco-Navarro, & Pérez-Sacristán, 2011), the main goal of this study was investigating anti-proliferative effects and mechanistic aspects of the action of β-Cx and β-sitosterol, campesterol and stigmasterol (alone or in combination) in a human colon cancer cell line, at concentrations consistent with their serum levels after dietary ingestion. Caco-2 cells, widely used to check and compare the activity of natural or synthetic molecules, have been chosen. Although a few data report on the activity and mechanism(s) of action of β-Cx and Ps in colon cancer prevention, there is no information regarding effects of the simultaneous presence of these compounds in relation to colon cancer. The eventual antiproliferative effects of the combined compounds could suggest complementary activities to the well-known hypocholesterolemic effect and bone health actions of Ps and β-Cx, and consequently amplify their conventional uses as functional ingredients in the manufacture of functional foods.

2. Materials and methods

2.1. Reagents

β-sitosterol, stigmasterol, 2′,7′-dicholofluorescein diacetate (DCFDA), dimethyl-sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), 5,5′-dithiobis-(2-nitrobenzoic acid), propidium iodide (PI), RNase A, as well as all solution reagents for Western blot analysis, were from Sigma Chemical Co (St. Louis, MO, USA). β-cryptoxanthin was from Extrasynthese (Genay Cedex, France). Campesterol was from Steraloids (Newport, RI, USA). Annexin V apoptosis detection kit FITC was from eBioscience (San Diego, CA, USA). 3,3′-dihexyloxacarbocyanine iodide was from Molecular Probes (Eugene, OR, USA). Dulbecco's Modified Eagle Medium (DMEM + GlutaMAX™), fetal bovine serum, non-essential amino acids, HEPES, antibiotic solution (penicillin–streptomycin), phosphate buffered solution (PBS) and trypsin–EDTA solution (2.5 g/L trypsin and 0.2 g/L EDTA) were from Gibco (Scotland, UK). β-actin, calcium Fluo-3/AM fluorescent probe, poly (ADP-ribose) polymerase (PARP) monoclonal antibody (clone D-1) and phospho-Bad polyclonal affinity-purified antibody (clone Ser 136) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-IgG antibody was from DAKO (Glostrup, Denmark).

2.2. Cell culture and treatments

Undifferentiated human colon adenocarcinoma (Caco-2) cells (American Type Culture Collection, LGC Promochem, Italy), between passages 33 and 47, were grown in 75 cm² Falcon flasks in DMEM + GlutaMAX™ containing 4.5 g/L glucose and

supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) HEPES, 1% (v/v) and antibiotic solution (penicillin–streptomycin). Cells were incubated at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

In all experiments, cells were sub-cultured after trypsin treatment (2.5 g/L trypsin and 0.2 g/L EDTA) and seeded onto 96-well plates for viability assays and on 24-well plates for the rest of assays, at a density of 5×10^4 cells/cm², with 0.2 mL and 1 mL of DMEM, respectively. Following 24 h from seeding, the cells were treated 24 h with β-Cx and/or major dietary Ps, alone or in combination (as the sum of the concentrations of each individual phytochemical alone) (Ps mix – only phytosterols – or β-Cx + Ps mix), at concentrations compatible with physiological serum concentrations [β-Cx (3 μM), β-sitosterol (12 μM), campesterol (1 μM), stigmasterol (0.25 μM)] obtained after consumption of beverages made of fruit juice and milk that contained all these phytochemicals (Cilla et al., 2011; Granado-Lorencio et al., 2011, 2014). Solutions of phytochemicals were in DMEM containing a maximum of 1.25% (v/v) of ethanol in all assays. Control cultures were exposed to DMEM containing ethanol (1.25%, v/v). At the applied conditions, ethanol did not affect cell viability. Cell viability was measured according to the MTT method (Mosmann, 1983).

2.3. Cell cycle analysis

Cell cycle stage was analyzed by flow cytometry. Aliquots of 1×10^6 cells were harvested by centrifugation, washed with PBS and incubated in the dark in a 5 mM Na–phosphate buffer solution containing triton (0.1%, v/v), 20 μg/mL PI and 200 μg/mL RNase, for 30 min at 4 °C in the dark. Then, samples were immediately subjected to cytofluorimetric analysis by Epics XL™ flow cytometer using Expo32 software (Beckman Coulter, Miami, FL, USA). At least 1×10^4 cells were analyzed for each sample.

2.4. Measurement of apoptosis through phosphatidylserine exposure

Flow cytometry by double staining with Annexin V/propidium iodide (PI) was used to detect externalization of phosphatidylserine to the cell surface. Cells were adjusted to 1×10^6 cells/mL with binding buffer. Cell suspension (100 μL) was added to a new tube, and incubated with 5 μL Annexin V and 10 μL of 20 μg/mL PI solution at room temperature, in the dark for 15 min. Then, samples of at least 1×10^4 cells were subjected to FACS analysis by appropriate two-dimensional gating method.

2.5. Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential (Δψm) was assayed by flow cytofluorimetry as reported (Tesoriere, Attanzio, Allegra, Gentile, & Livrea, 2013), using cationic lipophilic dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) that accumulates in the mitochondrial matrix. Changes in mitochondrial membrane potential are indicated by a reduction in $DiOC_6$-induced fluorescence intensity. Cells were incubated with $DiOC_6$ at a 40 nmol/L final concentration, for 15 min at 37 °C. After centrifugation, cells were washed with PBS and resuspended in 500 μL PBS. Fluorescent intensities were analyzed in at least 1×10^4 cells for each sample.

2.6. Measurement of intracellular reactive oxygen/nitrogen species (RONS)

DCFDA dye is commonly used to measure the cell oxidative status through a rapid quantitation of RONS. RONS level was monitored by measuring fluorescence changes that resulted from intracellular oxidation of DCFDA. DCFDA at 10 μM final concentration was added to the cell medium. Then cells were collected by centrifugation for 5 min at 480 g at 4 °C, washed, suspended in PBS and subjected to FACS analysis. At least 1×10^4 cells were analyzed for each sample.

2.7. Measurement of cytosolic calcium

Intracellular Ca^{2+} concentration was measured using fluo-3/AM as a fluorescent Ca^{2+} probe, whose intensity is directly representative of cellular concentration of the ion. Fluo-3/AM at 2 μM final concentration was added to the cell medium and cells incubated in the dark, 30 min, 37 °C. After centrifugation (480 g/5 min), cells were washed with PBS and suspended in 500 μL PBS. The fluorescent intensities were analyzed by FACS analysis in at least 1×10^4 cells for each sample.

2.8. Measurement of cellular thiols

Protein thiols and reduced glutathione were measured spectrophotometrically as reported (Tesoriere et al., 2013). After treatment, cells were collected by centrifugation (480 g/5 min), washed twice with cold PBS containing 0.1 M butylated hydroxytoluene and lysed by sonication. Cell lysates were mixed with 10% SDS and 30 μM of 5,5′-dithiobis-(2-nitrobenzoic acid) and incubated with shaking at room temperature for 30 min. The total amount of reduced thiols, including both protein thiols and glutathione, was measured spectrophotometrically at 412 nm.

2.9. Western blot analysis

After treatment, cells were collected by centrifugation, washed twice with cold PBS and gently lysed for 60 min in ice-cold lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mM benzamidine and 0.5 mM dithiothreitol (DTT)). The lysates were centrifuged at 13,000 g for 5 min and supernatants (cytosolic fraction) were immediately portioned and stored at –80 °C up to 2 weeks. The nuclear pellet was resuspended in 60 μL of high-salt extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mM benzamidine and 0.5 mM DTT) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 g and the supernatant was portioned and stored at –80 °C. The protein concentration of each sample was determined by using the Bradford protein assay reagent (Bio-Rad, Segrate, Milano, Italy). Protein samples (30 μg/line) were separated on 12%

SDS-PAGE and transferred to nitrocellulose membrane. The immunoblot was incubated overnight at 4 °C with blocking solution (5% skimmed milk), followed by incubation with anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibody or anti-phospho-Bad-polyclonal affinity-purified antibody for 1 h at room temperature. Blots were washed two times with Tween 20/Tris-buffered saline and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated anti-IgG antibody for 1 h at room temperature. Blots were again washed five times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (ChemiDoc XRS, Bio-Rad,). Immunoreactions were also performed using β-actin antibody as loading controls.

2.10. Statistical analysis

Results are given as means and standard deviations. Three independent observations were carried out for each experiment, replicated three times. One-way analysis of variance (ANOVA) and the LSD *post-hoc* test were applied to determine differences between treated and control cells. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.0 (Rockville, MA, USA) was used for the statistical analysis.

3. Results

3.1. Impact of β-Cx and/or Ps on viability and apoptosis

Effects on Caco-2 cell growth after 24 h treatment with three concentrations (low, medium and high) of β-Cx and/or dietary Ps, alone or combined, were assessed by MTT assay (Fig. 1A). The absence of cell detachment was checked with the microscope before the assays to assure that only attached cells were determined. With the exception of β-Cx and campesterol at their lowest concentrations, all other treatments caused a significant decrease in cell viability (21–44%), although a clear dose–response effect was not evident. In addition, no additive or synergistic effect was observed for the mixtures Ps-mix and β-Cx + Ps-mix. Rather, a trend, toward a lower anti-proliferative effect was observed with mixtures containing higher amounts of phytochemicals compared to β-Cx, campesterol and stigmasterol alone at their highest concentration. From these data, subsequent experiments intended to unravel possible mechanism(s) underlying anti-proliferative activity were carried out with the medium concentrations of all compounds.

Cytofluorimetric analysis after cell staining with PI was used to evaluate cell cycle. As compared with control cells, all treatments exerted a significant effect on cell cycle progression (Fig. 1B). The presence of a significantly higher proportion of cells in sub-G1 phase (considered a marker of cell death by DNA fragmentation associated with apoptosis) (Choi et al., 2003) was observed with all treatments, with the exception of β-sitosterol. The latter, however, exerted a significant increase in G2/M phase (+16% vs. control), which could be related to DNA damage (Nagahama et al., 2014) indicating that activation of other check points regulating cell mitotic phases is involved in the apoptotic mechanism of this Ps. The induction of a sub-G1 cell

population versus control was higher for Ps-mix (+145%) and stigmasterol (+134%), followed by β-Cx + Ps-mix (+93%) and campesterol (+92%), with β-Cx (+36%) showing the lowest effect. These results again indicated absence of synergistic effects of compounds in mixtures, and an apparent predominant action of stigmasterol.

Apoptosis indicators, such as i) externalization of plasma membrane phosphatidylserine, ii) changes in mitochondrial transmembrane potential and iii) PARP-1 cleavage were evaluated. Cytofluorimetric analysis of Annexin V-FITC and PI double labeled Caco-2 cells was carried out to determine phosphatidylserine externalization, an event occurring in the early phase of apoptotic cell death when cell membrane is still intact (Fig. 2) (Annexin+/PI−, lower right quadrant). In late apoptosis, a damaged cell membrane allows PI to enter and stain DNA (Annexin+/PI+, upper right quadrant). All phytochemicals, alone or in mixture, induced a two-fold to four-fold increase of early apoptosis; in addition stigmasterol, Ps-mix and β-Cx + Ps-mix doubled the proportion of cells in late apoptosis, as compared with control (Fig. 2). Globally, the highest values were found for mixtures, again with a marked effect of stigmasterol. Loss of mitochondrial trans-membrane potential, considered a hallmark of apoptosis during early stages, was indicated by decreased $DiOC_6$ red fluorescence (Fig. 3A). All treatments caused a fall (not significant for β-sitosterol) of mitochondrial trans-membrane potential (31–36% vs control), without differences among them. The fact that β-sitosterol did not evoke a significant loss of mitochondrial transmembrane potential with elevated G2/M arrest may suggest a different mechanism for this compound, through extrinsic apoptotic pathway. Finally, cleavage of specific target proteins, such as PARP-1, by activated caspase-3 was investigated. Treatment of cells with the phytochemicals caused cleavage of PARP-1 (Fig. 3B), with accumulation of the 89 kDa fragment and a concomitant decrease of the full-length 116 kDa protein compared to controls. The effect was higher for β-sitosterol, followed by campesterol, stigmasterol and PS mix, with β-Cx and β-Cx + PS showing the lowest values.

3.2. Effect of β-Cx and/or Ps on cellular redox state

RONS accumulation in cancer cells may lead to cell death (Nair et al., 2007). The intracellular levels of RONS determined by flow cytometry (Fig. 4A), revealed an approximately ten-fold increase with all treatments compared to control cells, without appreciable differences among treatments. On the other hand, determination of total cell thiols (which includes GSH – the main non enzymatic intracellular antioxidant compound) (Fig. 4B) showed a significant increase (17–63%) of total thiols versus control, being higher for Ps-mix and lower for campesterol. Additive or synergistic effects of the mixtures were not observed.

3.3. Induction of intracellular Ca^{2+} elevation by β-Cx and/or Ps

Increase of cytoplasmic Ca^{2+} precedes events of the apoptotic cascade and can take place before elevation of RONS and changes in gene expression (Mackrill, 2011). Flow cytometry analysis using Fluo-3/AM staining (Fig. 5A) indicated a significant increase of cytosolic Ca^{2+}, except for campesterol,
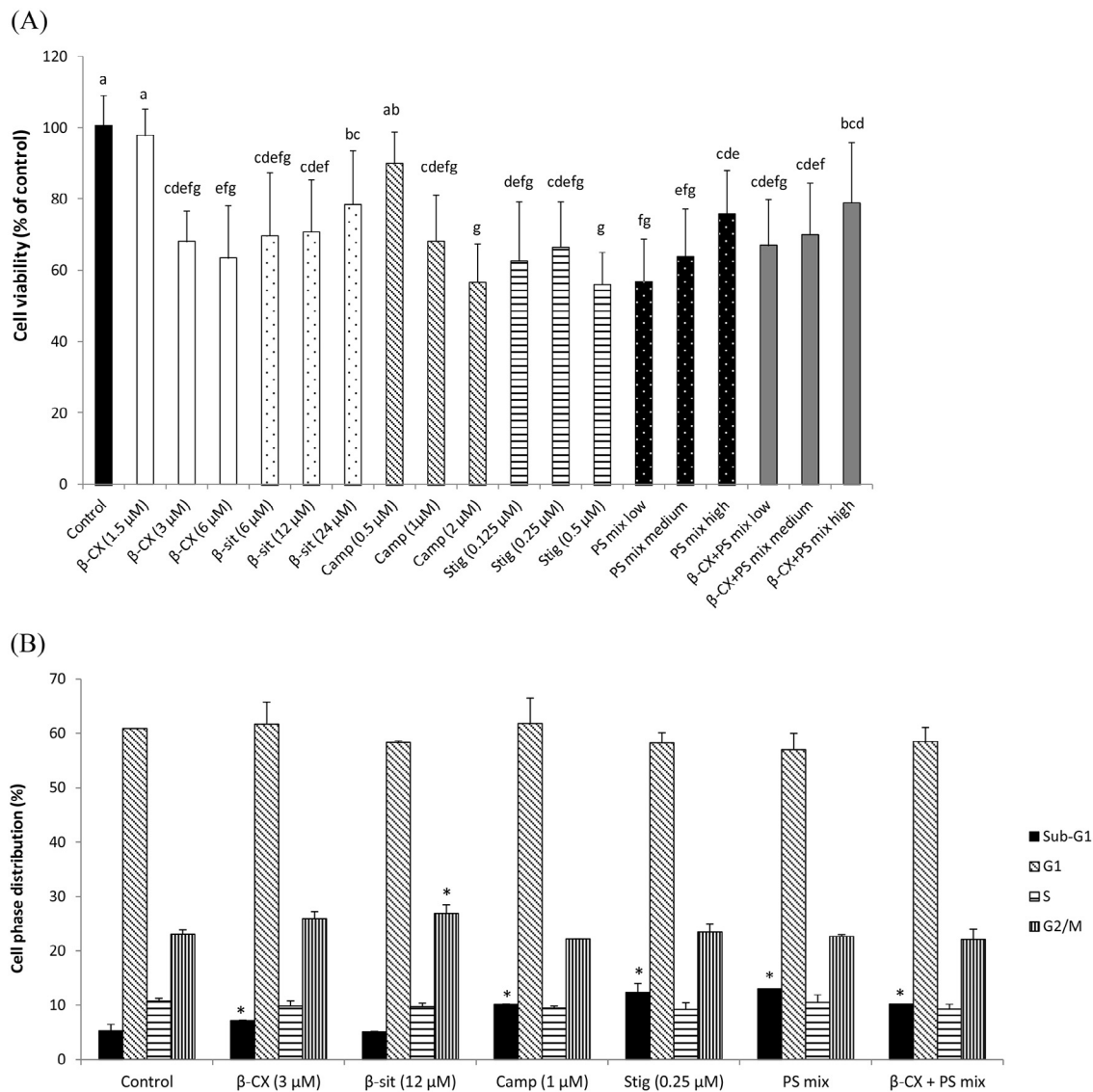
(A)

(B)

Fig. 1 – Effect of dietary β-Cx and/or major Ps (alone or in combination) on (A) viability (MTT assay) and (B) cell cycle distribution (flow cytometry analysis) of Caco-2 cells after 24 h treatment. Data are the means ± SD of three experiments carried out in triplicate. Different lowercase letters (a–g) in (A) indicate statistical significant differences among treatments. The presence of an asterisk in (B) denote statistical significant differences vs. control (p < 0.05); one-way ANOVA followed by LSD *post-hoc* test.

only showing a non-significant trend of rise. Ps-mix displayed the highest value, with a very nearly additive effect, whereas the presence of β-Cx in the mixture decreased the effect.

Dephosphorylated BAD acts as a pro-apoptotic factor that interacts with and inhibits the anti-apoptotic protein Bcl-2, a fact linked with intracellular Ca^{2+} elevation in early steps. Western blot analyses performed with phospho-BAD ser 136-specific antibody showed a significant decrease of the phosphorylated BAD level in comparison with control cells (Fig. 5B), being higher for β-sitosterol, campesterol and Ps-mix, followed by β-Cx, and then stigmasterol and β-Cx + Ps-mix with lowest values.

4. Discussion

Chemoprevention through dietary intervention may effectively control cancer development. In this context phytochemicals seem to be an attractive and cost-effective approach, especially considering the minimal effects for normal cells (Stadler & Ratain, 2000). Though regarded to have low potency when compared to pharmaceuticals, since they are ingested regularly and in significant amounts, dietary phytochemicals may have noticeable long-term effects (Espín, García-Conesa, & Tomás-Barberán, 2007). Taking into account that these compounds are ingested with foods containing many
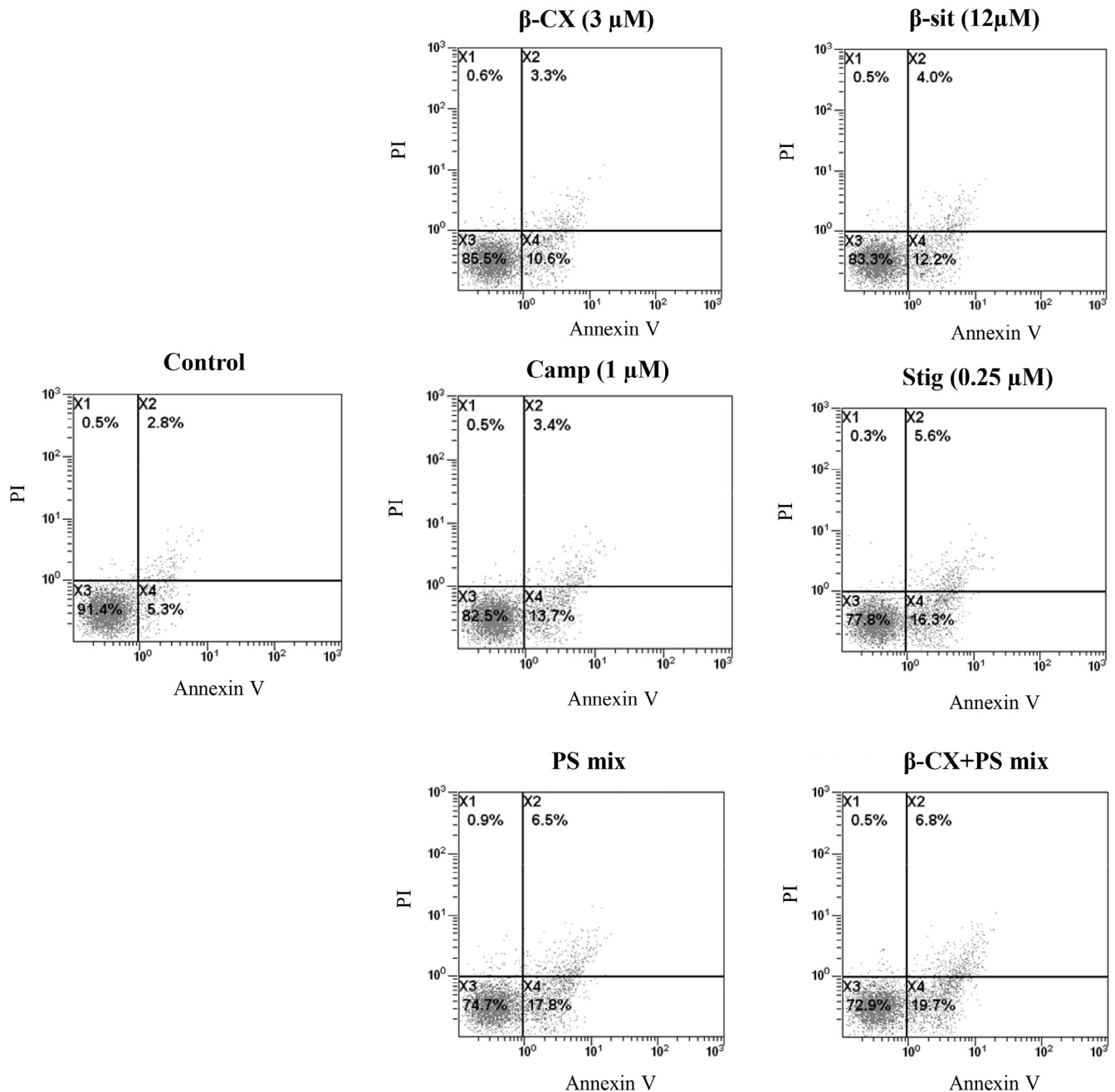
Fig. 2 – Effect of dietary β-Cx and/or major Ps (alone or in combination) on apoptosis of Caco-2 cells after 24 h treatment. Percentage of Annexin V/propidium iodide (PI) double-stained cells was evaluated by flow cytometry analysis. Data presented are representative images of three experiments in triplicate.

of them at one time, the present study investigated the xanthophyll β-Cx and the main dietary plant sterols, β-sitosterol, campesterol and stigmasterol either alone or, for the first time, in combination, in a model of human colon cancer, and provided evidence of their anti-proliferative effects, reducing cell growth up to 44%, under our conditions. Importantly, these effects were observed with concentrations of these phytochemicals compatible with the serum levels reported in humans after regular consumption of a functional beverage containing a mix of all these molecules (Cilla et al., 2011; Granado-Lorencio et al., 2011, 2014).

Mechanistic aspects of the anti-proliferative effects explored at a molecular level provided evidence that the arrest of growth was related to apoptotic cell death, possibly through the mitochondrial pathway. One of the early steps in this process is the increase of cytosolic Ca^{2+}, followed by a number of events downstream, including dephosphorylation of BAD, mitochondrial depolarization and caspase 3-dependent PARP cleavage (Mignotte & Vayssiere, 1999). All these events have been observed in our assays, thus providing a molecular basis to the decrease of cell viability through mitochondrial dysfunction, first suggested by the MTT assay, and to flow cytometry
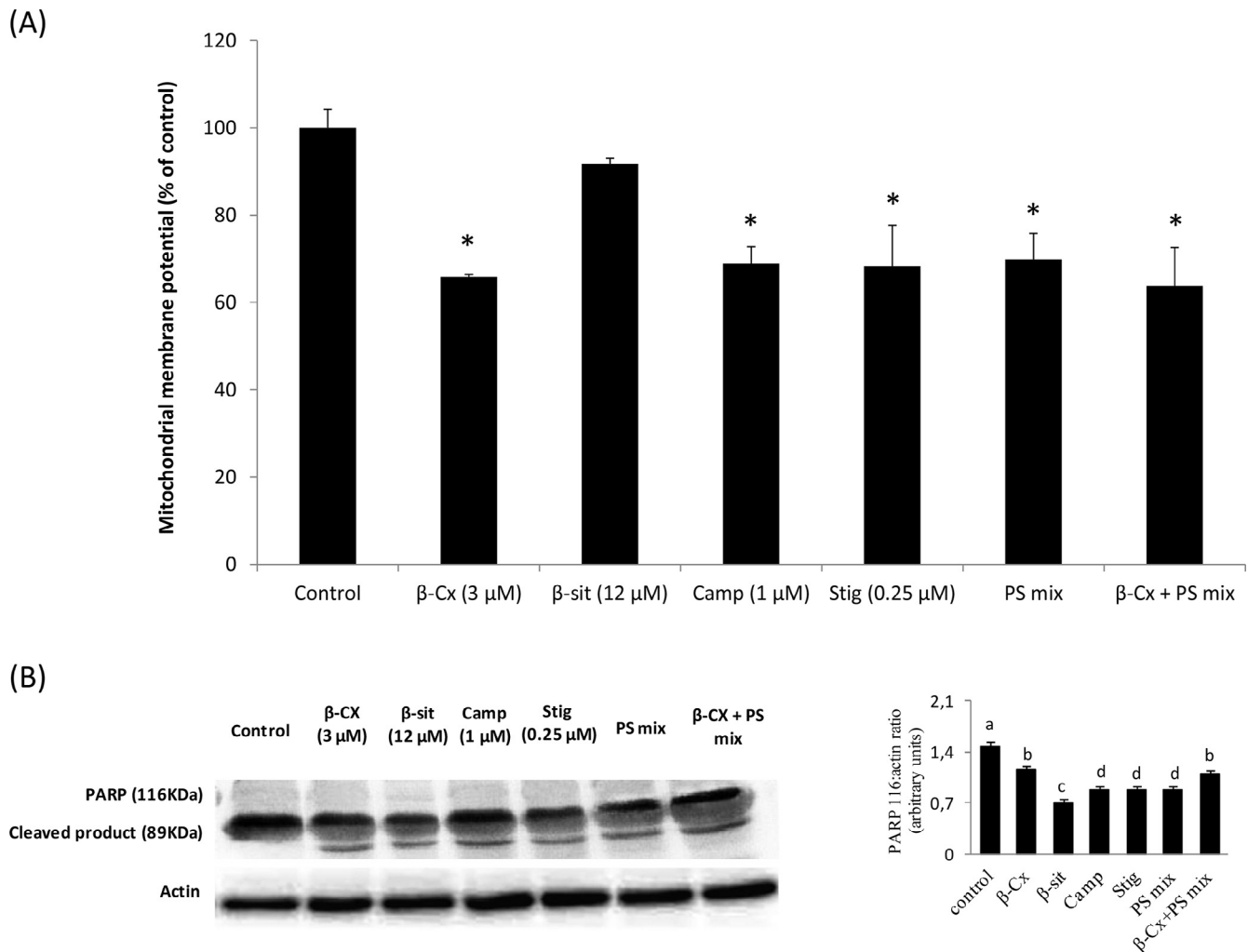
(A)

(B)

Fig. 3 – Effect of dietary β-Cx and/or major Ps (alone or in combination) on Caco-2 cell (A) mitochondrial trans-membrane potential (Δψm) and (B) poly(ADP-ribose) polymerase (PARP-1) cleavage, after 24 h treatment, determined by flow cytometry and Western blotting, respectively. (A) Data are the mean ± SD of three triplicate experiments. *Statistically significant differences (p < 0.05) versus control using one-way ANOVA followed by LSD *post-hoc* test. (B) Representative image of three separate experiments with comparable results. Densitometry data are the means ± SD. Mean values with different letters differ (p < 0.05; one-way ANOVA; LSD *post-hoc* test).

analysis that showed a rise of cell population in sub-G1 and presence of Annexin V-binding cells in early apoptosis. Interestingly, β-Sitosterol has been demonstrated to activate Fas, a cell surface death receptor whose activation constitutes the extrinsic apoptotic pathway, in both MCF-7 and MDA-MB-231 breast cancer cells; in addition, an alternative apoptotic signaling pathway for β-sitosterol in HT-29 colon cancer cells has been linked with a reduction in cellular sphingomyelin and an increase in ceramide levels, which are associated with the promotion of cell cycle arrest and apoptosis; thus possibly explaining the different behavior for this phytochemical (Bradford & Awad, 2010; Grattan, 2013).

Influx of calcium ions from the extracellular environment into cells involves opening of channels that are controlled through different ways, including oxidative stress (Bogeski et al., 2011). Oxidative stress-related cytosolic Ca^{2+} elevation has been considered to cause Ca^{2+} influx into mitochondria and into nuclei, this disrupting normal oxidative metabolism and

modulating gene transcription and nucleases that control apoptosis (Ermak & Davies, 2002). In agreement with the potential of oxidative stress and calcium elevation in the progress of the apoptotic process, we measured a remarkable RONS production associated with the activity of our compounds, a finding associated with the apoptotic activity of β-sitosterol in multiple myeloma U266 cells (Sook et al., 2014). In this context it may be considered that intracellular RONS itself may be a trigger of apoptosis, since excess RONS generated by phytochemicals can directly oxidize and activate one or more protein factors involved in this process (Khan et al., 2014). It should be mentioned that in normal cells, this scenario would lead to disruption of cell redox homeostasis and to oxidant environment; however, this is not a rule in cancer, and in our Caco-2 cells we observed an increase of total thiols (protein-SH and GSH) after treatment with the compounds, co-existent with RONS generation. A high level of GSH is often observed in many human cancer cells, assumed to be a defense or an adaptive
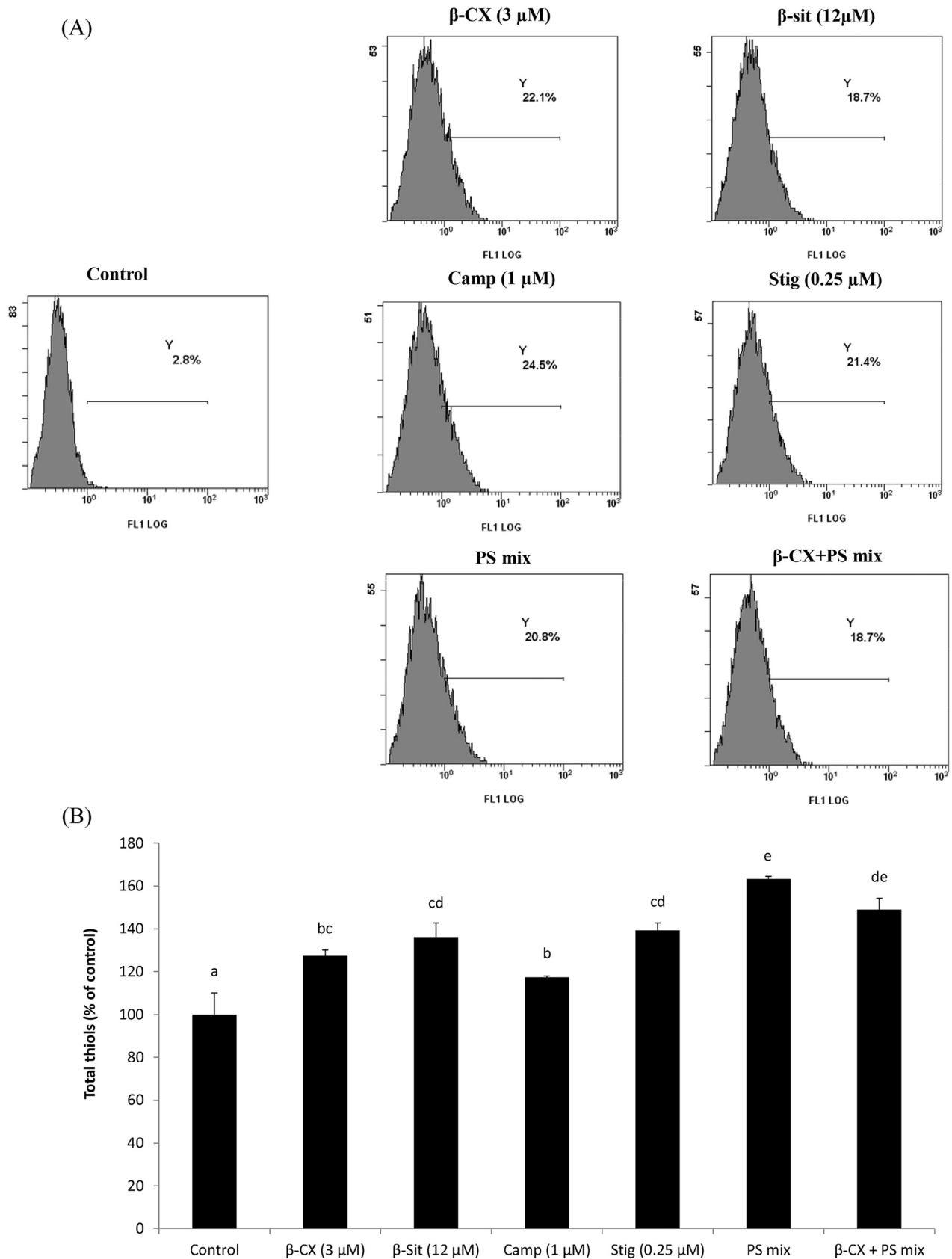
(A)

(B)

Fig. 4 – RONS production (A) and total cellular thiols (B) in Caco-2 cells after 24 h treatment with dietary β-Cx and/or major Ps (alone or in combination), determined by flow cytometry and spectrophotometry, respectively. RONS data are representative images of three triplicate experiments with comparable results. Mean values with different lowercase letters (a–e) differ ($p < 0.05$; one-way ANOVA; LSD *post-hoc* test).
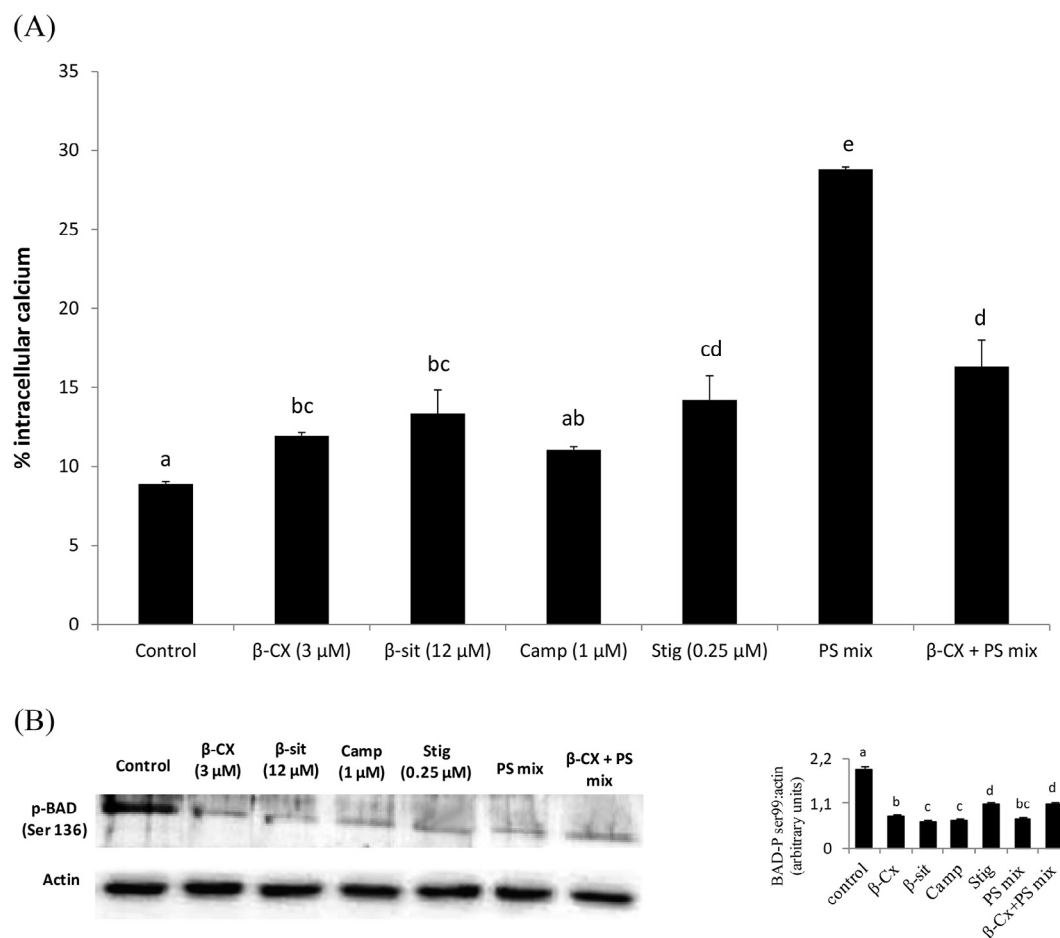
(A)

(B)

Fig. 5 – Effect of dietary β-Cx and/or major Ps (alone or in combination) after 24 h treatment of Caco-2 cells, on (A) intracellular Ca^{2+} level by flow cytometry and (B) expression of p-Bad by Western blotting in undifferentiated Caco-2 cells. Representative image (B) of three separate experiments with comparable results. Densitometry data are the means ± SD. Mean values with different lowercase letters (a–e) differ (p < 0.05; one-way ANOVA; LSD *post-hoc* test).

response against oxidative stress (Priya, Nigam, Bajpai, & Kumar, 2014), but in our case not enough to overcome the apoptotic fate caused by the treatment with phytochemicals. In addition, it should be mentioned that the total protein increased 1.5–2.5 folds in Caco-2 cells after all treatments (not shown), indicating a response in term of protein expression.

With the exception of the very nearly additive effect observed with the Ps-mix in the rise of intracellular Ca^{2+} levels, additive or synergistic anti-proliferative effects were not observed in our cell system when Ps and/or β-Cx were combined. Mechanisms leading cancer cells to grow or, inversely, modify pathways to revert proliferation and trigger death programs are largely unknown. Key molecular devices existing in cells are possibly to be stimulated and/or restored and go to the desired outcome by starting specific pathways. If bioactive phytochemicals, share a common mechanism of action, provided the amount necessary to trigger the response, the effect of their combination could not be higher than the individual one. Indeed in our experiments, high levels of cytosolic calcium essential to trigger an apoptotic response have appeared associated with the activity of all phytochemicals, either individual or in mixtures, and the response in term of anti-proliferative activity were not significantly different. In many cases an

apoptotic factor may be expressed as a result of the phytochemical activity – which is often linked to redox variations of the environment, e.g. by RONS production. Were this the case, again, we would have observed, as we did, RONS production as a consequence of all treatments, individual compounds and mixtures, with small differences between them. Finally, when diverse phytochemicals act together, the possibility that they may also work against each other cannot be ruled out. Varying effects from the combination of the Ps utilized in our work can be suggested from studies reporting quite different behavior of these compounds on the same cell system. Stigmasterol suppressed, but β-sitosterol promoted, and campesterol did not affect, cytokine secretion in macrophages, indicating a sterol type-specific activity (Sabeva et al., 2011). In another example close to the methodology of our study, the combination of β-sitosterol and resveratrol showed no additive or synergistic effect, rather caused decrease of the cell growth inhibition of human prostate cancer PC-3 cells. β-sitosterol was more effective than resveratrol in inducing apoptosis, and the combination had an intermediate effect after 1 day of treatment (Awad, Burr, & Fink, 2005). Then, the phytochemicals considered in the present study could have some antagonistic action when they play together. Nevertheless, our mixtures

still displayed an anti-proliferative activity comparable with the individual compounds, and also some of the best results in terms of cell cycle sub-G1 rise, early apoptosis and intracellular Ca^{2+} accumulation, pointing out to the beneficial effects derived from this combination.

Beneficial activity of β-Cx and PS has been suggested in several studies carried out with various cancer cells of the gastro-intestinal (GI) tract, under a number of experimental conditions. In agreement with our results, mandarin citrus juices and pulp rich in β-Cx and hesperidin have shown suppressive and preventive activity in colon cancer in male F344 rats (Tanaka et al., 2000, 2012) and in human colon cancer Colo 320 and Colo 205 cells (Ugocsai et al., 2005). In the case of colon cancer cells, treatment with β-Cx at 10 μg/mL (≈18 μM) for 24 h caused 4.6% (Colo 320) and 11% (Colo 205) early apoptosis (Ugocsai et al., 2005), an effect lower than we observed in Caco-2 cells with a 24 h treatment with 3 μM β-Cx, resulting in a 11% early apoptosis. β-Cx has also been shown to be active in other cells lining in the GI tract (BGC human stomach cancer cells), where a 72 h treatment with 0.01–20 μM dose-dependently decreased cell proliferation (≈up to 70%) and induced cell cycle arrest at G0/G1 phase, without sub-G1 population (Wu et al., 2013). In tune with our results, anti-proliferative activity of PS in colon cancer has been reported, with individual compounds at variable concentrations (2.5 to 400 μM) and times of exposure (24 to 72 h), in cell lines such as HT-29 (Awad et al., 1996; Jayaprakasha et al., 2010; Montserrat-de la Paz et al., 2015), HCT116 (Choi et al., 2003), Caco-2 (Daly et al., 2009) and Colo 320 (Baskar et al., 2010). In these studies β-sitosterol showed anti-proliferative effects between 2.5 and 20 μM in HT-29 and HCT116 cell lines (Awad et al., 1996; Choi et al., 2003), but differently from our data only pharmacological doses of β-sitosterol (200 and 400 μM) and campesterol (400 μM), for 48 h, decreased viability of Caco-2 cells by 39–47 and 21%, respectively, without significant apoptotic potential at 25 μM (Daly et al., 2009). The different initial cell density used for the assays and methodology employed in MTT assay (20 h incubation with solubilization solution) could partly explain these discrepancies. In other studies, β-sitosterol (15–240 μM) induced a significant dose-dependent growth inhibition of Colo 320 cells and induced apoptosis by scavenging reactive oxygen species (Baskar et al., 2010), and β-sitosterol glucoside from sour orange evoked a 38% decrease of HT-29 cell viability, when cells were treated with 40 μM for 24 h (Jayaprakasha et al., 2010). Furthermore, β-sitosterol (4–32 μM) significantly inhibited the growth and induced apoptosis in SGC-7901 human stomach cancer cells when treated for 120 h (Zhao, Chang, Qu, Li, & Cui, 2009). In addition, phytosterols isolated from evening primrose oil and its main components β-sitosterol and campesterol (100 μM) showed a decrease in cell proliferation, apoptosis induction and arrest in G2/M cell cycle (β-sitosterol) for 48 h in HT-29 cells (Montserrat-de la Paz et al., 2015). To the best of our knowledge, no report exists for stigmasterol in colon cancer cells. However, stigmasterol isolated from *Navicula incerta* marine algae induced apoptosis in human hepatocarcinoma HepG2 cells treated for 24 h with 5 to 20 μM (Kim, Li, Kang, Ryu, & Kim, 2014). All these works highlight the potential of Ps as anti-carcinogenic natural compounds against colon cancer and show the variable sensitivity of distinct colon cancer cell lines to these bioactive compounds. However, none of those studies have

addressed the anti-proliferative beneficial activity of these phytochemicals when assayed in mixtures, which would deserve to be done in the light of the diverse sensitivity of cancer cells to phytosterols. On the other hand, mixtures comparable with that used in the present work have to be assayed in intestinal cancer cells other than Caco-2 to have a more complete picture of their activity and possible applications in fortified and functional foods.

5. Conclusions

β-Cx and main dietary Ps (β-sitosterol, campesterol and stigmasterol) alone or in combination, at concentrations compatible with physiological human serum levels, have shown anti-carcinogenic activity against human colon adenocarcinoma Caco-2 cells through the mitochondrial pathway of apoptosis, due to increase of cytosolic Ca^{2+} and oxidative stress. This highlights the importance of performing bioactivity assays when deciding about the activity of mixed dietary phytochemicals and potential health effects of functional foods. β-Cx and Ps did not show additive nor synergistic effects, however they retained the same anti-proliferative activity as the individual compounds when combined, indicating that antagonistic actions at the applied concentrations did not occur. The anti-proliferative activity in colon cancer cells observed in our *in vitro* study may be overlapping and complementary to the cardiovascular and osteoporosis decreasing risk previously reported after the intake of functional beverages containing Ps and β-Cx.

Acknowledgements

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