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**Apoptotic effect of a phytosterol-ingredient and its main phytosterol (β-sitosterol) in human cancer cell lines**

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**ABSTRACT**

Dietary interventions may effectively control cancer development, with phytosterols (PS) being a class of cancer chemopreventive dietary phytochemicals. The present study, for the first time, evaluates the antiproliferative effects of a PS-ingredient used for the enrichment of several foods and its main PS, β-sitosterol, at physiological serum levels, in the most prevalent cancer cells in women (breast (MCF-7), colon (HCT116) and cervical (HeLa)). In all three cell lines, these compounds induced significant cell viability reduction without a clear time- and dose-dependent response. Moreover, all treatments produced apoptotic cell death with the induction of DNA fragmentation through the appearance of a sub-G1 cell population. Thus, the use of PS as functional ingredients in the development of PS-enriched foods could exert a potential preventive effect against human breast, colon and cervical cancer, although further in vivo studies are required to confirm our preclinical findings.

**INTRODUCTION**

The global burden of cancer has gained great attention, as it is among the leading causes of morbidity and mortality worldwide. It has been estimated that there were 14.1 million new cancer cases and 8.2 million deaths in 2012, and the numbers are expected to rise significantly over the coming decades. In women, the most prevalent cancers are breast cancer (25.2%), followed by colorectal cancer (9.2%) and cervical cancer (7.9%) (Ferlay et al. 2015). Bioactive food components such as phytosterols (PS) could constitute a new alternative as cancer chemopreventive and therapeutic agents against these kinds of cancers, intervening in several regulatory pathways. Different reviews on the anticancer effects of PS (mainly β-sitosterol) have been published (Woyengo et al. 2009; Bradford and Awad 2010; Shahzad et al. 2017). Moreover, it has been reported that PS do not alter the viability and the normal cell growth of non-cancerous cells such as differentiated Caco-2 (a model of normal small intestinal cells) (Awad et al. 2005), COS-1 (fibroblast cells) (Jayaprakasha et al. 2007), VERO (monkey kidney cells) (Baskar et al. 2010) or MRC-5 cells (fibroblast derived from lung cells) (Rahman et al. 2013) in a range of concentrations (0.6–1000 μM) in which are our own tested concentrations (13–52 μM).

The antiproliferative activity of β-sitosterol standards has been studied in breast cancer (MCF-7) (Awad et al. 2007, 2008; Rubis et al. 2010), colon cancer (HT-29, Caco-2 and/or HCT116) (Awad et al. 1996, 1998; Choi et al. 2003; Daly et al. 2009; Montserrat-de la Paz et al. 2015) and cervical cancer (HeLa) cells (Cheng et al. 2015). Likewise, the antiproliferative activity of β-sitosterol standards has been studied in breast cancer (MCF-7) (Awad et al. 2007, 2008; Rubis et al. 2010), colon cancer (HT-29, Caco-2 and/or HCT116) (Awad et al. 1996, 1998; Choi et al. 2003; Daly et al. 2009; Montserrat-de la Paz et al. 2015) and cervical cancer (HeLa) cells (Cheng et al. 2015). Likewise, the anticarcinogenic effects of isolated PS from plant extracts have also been evaluated in MCF-7 (Chai et al. 2008; Malek et al. 2009; Rahman et al. 2013; Yaacob et al. 2015; Tahsin et al. 2017), in HCT116 (Malek et al. 2009; Rahman et al. 2013) and in HeLa cells (Block et al. 2004; Csupor-Loffler et al. 2011; Hamdan et al. 2011; Han et al. 2013). Since dietary PS alone are unable to offer the recommended daily doses (1.5–3 g/day) for lowering LDL-cholesterol (Gylling et al. 2014), several foods are currently enriched with PS. The main sources of PS used for this purpose are vegetable oil deodoriser distillate and tall oil (a byproduct of the kraft pulping of...
wood) (García-Llatas and Rodríguez-Estrada 2011; González-Larena et al. 2011).

Previous studies conducted by our research group have reported a positive effect on cardiovascular risk and bone turnover markers after the regular consumption of milk-based fruit beverages enriched with PS from tall oil (Granado-Lorencio et al. 2014); anticarcinogenic effects of PS standards (β-sitosterol, campesterol and stigmasterol, alone or combined) upon Caco-2 cells, at concentrations compatible with physiological serum levels after the intake of such beverages (Cilla et al. 2015), and cytoprotective effects of the bioaccessible fractions obtained after simulated gastrointestinal digestion of these beverages in Caco-2 cells (López-García et al. 2017a). Moreover, due to the fact that PS undergo less absorption (0.5–2%) (Gylling et al. 2014), these compounds may reach the colon and exert local actions at gastrointestinal level. In this context, a recent study carried out by our research group (López-García et al. 2017b), considering the estimated colonic concentrations of PS capable of reaching the colon after the intake of the aforementioned kind of beverage, revealed antiproliferative effects upon the Caco-2 cell line. However, the anticarcinogenic activity of the tall oil PS-ingredient used for food enrichment has not been evaluated so far. Thus, the present work, for the first time, studies the antiproliferative effect of a PS-ingredient from tall oil used for the enrichment of milk-based fruit beverage and its main PS (β-sitosterol) at concentrations compatible with physiological serum levels, following its regular consumption, upon human breast (MCF-7), colon (HCT116) and cervical cancer (HeLa) cell lines. In this regard, the present study could complement the well-known hypocholesterolemic effect of PS and moreover contribute to extend their use as functional ingredients in the development of PS-enriched foods to maximise their functionality.

**Materials and methods**

**Reagents**

Dimethyl-sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), (24R)-ethylcholsest-5-en-3β-ol (β-sitosterol), RPMI-1640 medium, propidium iodide (PI) and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). Annexin V apoptosis detection kit FITC was from eBioscience (San Diego, CA). Antibiotic solution (penicillin–streptomycin), nonessential amino acids, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), phosphate buffered solution (PBS) and trypsin–EDTA solution (2.5 g/l trypsin and 0.2 g/l EDTA) were obtained from Gibco (Scotland, UK).

Phytosterol powder free microcrystalline ingredient from tall oil, previously characterised by our research group (β-sitosterol 78.86%; sitostanol 11.95%; campesterol 7.13%; campestanol 1.20% and stigmasterol 0.82%; González-Larena et al. 2011), containing maltodextrin, sucrose ester and inulin was purchased from Lipofoods, Barcelona, Spain (Lipohytol® 146 ME Dispersible).

**Cell culture and treatments**

The human breast (MCF-7), colon (HCT116) and cervical cancer (HeLa) cells (American Type Culture Collection, LGC Promochem, Italy) were used between passages 33 and 47, and were grown in 75 cm² Falcon flasks in RPMI-1640 medium containing 4.5 g/l glucose and supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) nonessential amino acids, 1% (v/v) HEPES and antibiotic solution (penicillin–streptomycin). Cells were incubated in a humidified atmosphere (37 °C, 5% CO₂).

In all experiments, cells were seeded at a density of 5 × 10^4 cells/cm² after trypsin treatment (2.5 g/l trypsin and 0.2 g/l EDTA). For viability assays, cell lines were seeded during 24 h onto 96-well plates with 0.2 ml of RPMI medium, followed by treatment for 24 h or 48 h with the tall oil PS-ingredient or β-sitosterol at different concentrations (13, 26 and 52 μM). These concentrations are included in the range of PS physiological serum levels (5–30 μM), reaching values of 50 μM after the consumption of PS-enriched foods (Daly et al. 2009; Gylling et al. 2014). The lowest PS concentration assayed in the present work (13 μM), is similar to the serum β-sitosterol concentration (15 μM) obtained after the consumption of milk-based fruit beverages enriched with the tall oil PS-ingredient previously evaluated by our research group (García-Llatas et al. 2015). In the case of the PS-ingredient, the selected concentrations were based on the main PS (β-sitosterol).

For cell cycle and apoptosis assays, cells were seeded onto 24-well plates with 1 ml of RPMI medium. Following 24 h from seeding, cell lines were treated with PS-ingredient (13 μM) or β-sitosterol at different concentrations (13, 26 and 52 μM) for 48 h, based on the cell viability results. Control cultures and PS-ingredient were prepared with RPMI medium and β-sitosterol in tetrahydrofuran (0.1%) for all assays. Under the above-mentioned conditions, tetrahydrofuran did not affect cell viability (data not shown).
**Cell viability assay**

Cells were seeded onto 96-well plates at a density of 18,000 cells per well, and were maintained under appropriate culture conditions (37°C, 5% CO₂) for 24 h. After 24 or 48 h of treatment with the three concentrations of PS-ingredient or β-sitosterol (13, 26 and 52 μM), 4 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (5 mg/ml) was added, followed by incubation at 37°C for 2 h. Then, the MTT reaction agent was removed and 100 μl of DMSO was added to allow the dissolution of purple formazan products, as previously described (Girasolo et al. 2014).

**Cell cycle analysis**

For cell cycle assay, each cell line was treated and incubated during 48 h (37°C, 5% CO₂) with samples. Aliquots of 1 × 10⁶ cells were harvested by centrifugation (125 × g, 5 min), and the cell pellet was washed with PBS and incubated in the dark (30 min at 4°C) in a 5 mM sodium phosphate buffer solution containing 20 μg/ml PI, Triton (0.1%, v/v) and 200 μg/ml RNase. DNA fluorescence was measured by cytofluorometry using an Epics XL™ flow cytometer with Expo32 software (Beckman Coulter, Miami, FL). The relative distribution of 1 × 10⁴ events was analysed for each sample as described by Cilla et al. (2015).

**Assessment of apoptosis through phosphatidylserine exposure**

The apoptosis measurement was carried out according to Cilla et al. (2015) through flow cytometry by double staining with Annexin V/PI to detect externalisation of phosphatidylserine to the cell surface. Each cell line was treated and incubated during 48 h (37°C, 5% CO₂) with samples, and then adjusted to 1 × 10⁶ 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 (15 min). Then, for each sample 1 × 10⁴ events were analysed by flow cytometry with the appropriate two-dimensional gating method.

**Statistical analysis**

The analysis of all samples was performed in triplicate. One-way analysis of variance (ANOVA), followed by LSD post hoc testing were applied to determine differences between treated and control cells on the same day of treatment. A paired t-test was used for the MTT assay to detect statistically significant differences between different time periods for one same treatment. A significance level of $p < .05$ was adopted for all comparisons, and the Statgraphics Centurion XVI statistical package (Statpoint Technologies Inc., Warrenton, VA) was used throughout.

**Results**

The effects of PS treatment with tall oil PS-ingredient or its main PS (β-sitosterol) upon cell viability, cell cycle and apoptosis were evaluated at physiological concentrations in cell lines corresponding to the most prevalent cancers in women: breast (MCF-7), colon (HCT116) and cervical (HeLa).

**MCF-7 cells**

The effect of PS-ingredient or β-sitosterol (13, 26, 52 μM) after 24 and 48 h of incubation upon MCF-7 cell growth, assessed by MTT assay, is shown in Figure 1(A). Only the phytosterol-ingredient at 13 μM and the β-sitosterol standards showed a significant ($p < .05$) decrease in cell viability, being greater at 48 h. The decrease in cell viability produced by the PS-ingredient (13 μM) was 17% or 53%, which was similar to the results for β-sitosterol (13, 26 and 52 μM), ranging from 19–22% or 50–67% at 24 and 48 h, respectively – with no statistically significant ($p < .05$) dose-response effect among all the concentrations tested (Figure 1(A)). For this reason, the following assays (cell cycle and apoptosis studies) (Figure 1(B and C)) were carried out only with the physiological PS-ingredient concentration (13 μM) and β-sitosterol (13, 26 and 52 μM) at 48 h.

MCF-7 cell staining with PI was used to evaluate the cell cycle at 48 h by flow cytometry (Figure 1(B)). The percentage of cells in the sub-G1 phase (considered as a marker of DNA fragmentation) (Choi et al. 2003) increased with all treatments versus MCF-7 control cells, following the order: β-sitosterol 13 μM (7.1-fold) > β-sitosterol 26 μM (6.3-fold) > PS-ingredient 13 μM (5.4-fold) > β-sitosterol 52 μM (3.5-fold). This was accompanied by a concomitant and statistically significant ($p < .05$) decrease, in the same order, for the other cell cycle phases (20–58%, 15–51% and 21–55% in phases G1, S and G2/M, respectively) (Figure 1(B)). These results suggest that the PS-ingredient and β-sitosterol could exert an antiproliferative effect through apoptosis involving the modulation of cell cycle progression.
Figure 1. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on (A) viability (MTT assay), (B) cell cycle and (C) apoptosis of MCF-7 cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a–c) or different capital letters (A–B) indicate statistically significant differences (p < .05) among the treatments after 24 or 48 h of incubation, respectively. #Denotes statistically significant differences between 24 and 48 h for a same treatment; Cell cycle assay: different lowercase letters (a–e) indicate statistical significant differences (p < .05) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.
The best marker for evaluating cell death through apoptosis is phosphatidylserine externalisation, an event occurring in the early phase of apoptotic cell death when the cell membrane is still intact. For this purpose, Annexin V-FITC and PI double labelling was carried out by flow cytometry. As shown in Figure 1(C), all treatments induced an increase in early apoptosis versus control MCF-7 cells, with the same behaviour of samples (PS-ingredient and β-sitosterol standard) as evaluated in the cell cycle assay. These results again indicated the absence of a dose–response effect upon MCF-7 cells.

**HCT116 cells**

The effect of PS-ingredient or β-sitosterol (13, 26, 52 μM) upon HCT116 cell growth after 24 and 48 h of incubation is shown in Figure 2(A). All treatments (except PS-ingredient at 26 μM after 24 h) induced a statistically significant (p < .05) decrease in cell viability with respect to control cells. The greatest viability reductions were observed with the PS-ingredient at 13 μM (36 or 46% at 24 and 48 h, respectively) and β-sitosterol at 13, 26 and 52 μM (27–42% or 44–49% at 24 and 48 h, respectively), though with no clear time- and dose-response effect (Figure 2(A)).

After 48 h of exposure, an increase in the percentage of cells in sub-G1 phase was observed with all treatments (Figure 2(B)), with a significant (p < .05) higher value for PS-ingredient at 13 μM and β-sitosterol at 13 and 26 μM (6.7–8.8 fold) versus β-sitosterol 52 μM (3.3-fold). At the same time, we observed a significant (p < .05) decrease only in phase G1 (19.4–23.9%), with the exception of β-sitosterol at 52 μM (Figure 2(B)). This behaviour was also observed after Annexin V/PI assay (Figure 2(C)), where all treatments significantly (p < .05) induced an increase in early apoptosis (12.9–20.9%) versus control cells (1%), following the order: PS-ingredient 13 μM ≥ β-sitosterol 13 μM ≥ β-sitosterol 26 μM > β-sitosterol 52 μM. These results again indicated the absence of a dose–response effect upon HCT116 cells, and that the effect of PS-ingredient on this cell line was due to the apparent predominant action of its main PS (β-sitosterol).

**HeLa cells**

In the HeLa cell line after 24 h of incubation, only PS-ingredient (52 μM) and β-sitosterol (13 μM) showed a significant (p < .05) decrease of 20.5–21.4% versus control cells (Figure 3(A)). However, at 48 h, the PS-ingredient at concentrations of 13 and 26 μM (22.6 and 34.1%, respectively) also induced a decrease in HeLa cell viability. Moreover, a greater decrease produced by PS-ingredient at 26 and 52 μM and β-sitosterol (13 μM) at 48 h versus 24 h of exposure was observed (Figure 3(C)). Regarding cell cycle assay (Figure 3(B)), and in contrast to the results obtained in the other studied cell lines, the greatest increase in the percentage of cells in the sub-G1 phase corresponded to PS-ingredient 13 μM (3.4-fold), followed by β-sitosterol 52 μM (1.9-fold) ≥ 26 μM (1.5-fold) ≥ 13 μM (1.3-fold) versus control cells, with a concomitant significant (p < .05) decrease in phase G1 (28, 8.4 and 4.6% for PS-ingredient 13 μM, β-sitosterol 52 and 13 μM, respectively). Moreover, treatment with PS-ingredient (13 μM) also induced a significant (p < .05) reduction in phases S and G2/M of 20.6 and 23.7%, respectively (Figure 3(B)). This behaviour was also observed after Annexin V/PI assay (Figure 3(C)), where PS-ingredient 13 μM showed the highest proportion of early HeLa cell apoptosis (24.5%) versus control cells, and a dose–dependent effect of β-sitosterol standards was observed (14.3–20.4%, with a greater apoptotic effect induced by β-sitosterol 52 μM.

**Discussion**

In general, as it will be shown below, the differences observed in comparison with our study may be due to: (i) the kind of bioactive compound studied (β-sitosterol standard with or without cyclodextrin complexation, PS mix instead of PS alone, or isolated PS from plant extracts); (ii) the use of different incubation times (1–9 days); (iii) different concentration of PS (0–400 μM); and (iv) the use of different cells lines.

**Breast cancer cells**

Regarding the β-sitosterol standards, the growth inhibition percentages observed at 13 μM for 48 h are within levels recorded in previous studies (29–81%) with β-sitosterol at 16 μM tested between 1 and 5 days of incubation (Awad et al. 2007, 2008; Rubis et al. 2010). On the other hand, it has been observed that β-sitosterol intake is associated to a greater probability of oestrogen receptor-positive (related to MCF-7) than oestrogen receptor-negative tumours, possibly explaining that MCF-7 cells may be more resistant to this compound (Touillaud et al. 2005; Grattan 2013). These authors suggested biological effects of PS upon oestrogen receptor regulation, indirectly influencing
cellular processes such as oestrogen metabolism, oestrogen receptor function and expression, since PS can modify the fluidity of cholesterol-rich cell membranes (without altering their integrity) and inhibit membrane-bound molecules. However, Ju et al. (2004) reported that the reduction of tumour size in ovariectomized athymic mice injected with MCF-7 cells after the addition of β-sitosterol glycoside or β-sitosterol:β-sitosterol glycoside (99:1) to the diet (0.2 and 10 g/kg diet, respectively) was independent of oestrogen signalling. These authors, therefore, suggested that PS may be beneficial for women with breast cancer, though the mechanism involved is unclear. Overall, based on the epidemiological data and studies carried out with cells or animals, it can be concluded that controversy exists regarding the role of PS as a mediator of breast cell growth through oestrogen pathways.

Several mechanisms have been proposed for clarifying the action of β-sitosterol (16 μM) in relation to breast cancer cell (MCF-7) proliferation, such as the activation of the extrinsic apoptotic pathway through increased activity of caspase 8 (1.9-fold increase versus control), and a 30% increase in first apoptosis signal receptor (Fas) (Awad et al. 2007). Moreover, an increase in ceramide (an intracellular modulator of cell growth), which suggests that β-sitosterol activates de novo ceramide synthesis by stimulating serine palmitoyl transferase activity (Awad et al. 2008) is also observed. Globally, these results confirm the apoptotic action of β-sitosterol, in agreement with our own results. Regarding cell cycle distribution, and in contrast to our findings, β-sitosterol (1 and 5 μM) did not induce significant changes in the cell cycle of MCF-7 cells at 24 h (Rubis et al. 2010), possibly because of the lower concentrations and shorter incubation times involved.

To the best of our knowledge, no other similar antiproliferative studies have been made with PS-ingredients; however, different studies have evaluated the antiproliferative effect of PS (mainly β-sitosterol or PS mixtures) isolated from plant extracts upon MCF-7 cells, mainly through the determination of IC50 (the PS concentration causing 50% inhibition of cell growth), which is directly dependent upon the dose and exposure time involved. There is controversy as to when a plant extract can be considered to have an active cytotoxic effect based on IC50. According to Tahsin et al. (2017), a plant extract is active with IC50 < 500 μg/ml, while Malek et al. (2009) indicate that a plant extract is regarded as active with IC50 ≤ 20 μg/ml following 48 to 72 h of incubation. Nevertheless, it is recognised that the significant or nonsignificant cytotoxicity reflected by a given IC50 is conditioned to the sensitivity of the cell line involved.

Generally, it has been reported that treatment with mixtures of PS (mainly β-sitosterol, campesterol and stigmasterol) from different plant extracts showed no antiproliferative effects upon MCF-7 cells at concentrations of 0.1–100 μg/ml and incubation times of 24, 48 and 72 h (Malek et al. 2009; Rahman et al. 2013; Yaacob et al. 2015). Unlike, the PS-ingredient used in our study, mainly comprising β-sitosterol, showed an antiproliferative effect (17% and 53% at 24 and 48 h, respectively) at the lowest concentration used (5.39 μg/ml, 13 μM). The antiproliferative effect observed with the PS-ingredient could be mainly attributable to the presence of β-sitosterol, since cell viability inhibition values consistent with our own results, have been reported with similar (10 μM) or higher (174 μM) concentrations of β-sitosterol at 72 h (Chai et al. 2008; Malek et al. 2009).

Colon cancer cells

As far as we are aware, only one other study (Choi et al. 2003) has been carried out with β-sitosterol standard (2.5–20 μM) in the HCT116 cell line, inducing slightly greater growth inhibition in a dose-dependent manner (50 and 75% with 7.5 and 20 μM, respectively) compared with our results referred to β-sitosterol standard at 48 h (44–49% at 13, 26 and 52 μM, with no clear dose-response). Using different colon cancer cell lines as HT-29, β-sitosterol standard (16 μM) complexed with cyclodextrin, and involving longer treatment periods (3–9 or 5 days), showed similar (~35–67%) (Awad et al. 1996) or lower (19%) (Awad et al. 1998) antiproliferative responses than in our own study (27–49% at 13 μM and at 24 and 48 h). Our results are also within the range of antiproliferative values (~10–50%) reported by Montserrat-de la Paz et al. (2015) with β-sitosterol standard (0–100 μM) at 24 and 48 h upon HT-29 cells. Regarding Caco-2 cells, Daly et al. (2009) found that higher doses of β-sitosterol standard (200 and 400 μM) are necessary in order to obtain similar antiproliferative values (39–47%) at 48 h to our own results. In the same cell line, using serum (6, 12 and 24 μM) (Gilla et al. 2015) or estimated colonic concentrations (115 μM) (López-García et al. 2017b) of β-sitosterol standard or PS mix standards (β-sitosterol, campesterol and stigmasterol) (serum: 6.6, 13.2, 26.5 μM or colonic: 132 μM, respectively), was recorded a decrease in cell viability (21–44% or 57–59%, respectively), similar to that found in our
Figure 2. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on (A) viability (MTT assay), (B) cell cycle and (C) apoptosis of HCT116 cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a–d) or different capital letters (A–D) indicate statistically significant differences (p < .05) among the treatments after 24 h or 48 h of incubation, respectively; Cell cycle assay: different lowercase letters (a–c) indicate statistical significant differences (p < .05) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.
study at 24 h. Moreover, in agreement with our own findings, no clear dose-response relationship was observed between the different concentrations studied, with no evident additive or synergistic effects for the PS mix.

In general, HT-29 and Caco-2 cells seem to be more resistant to antiproliferative PS action than HCT116 cells, since the former needed longer times or higher PS concentrations than in our study to reach the same effects in the latter cells. This may be

Figure 3. Effect of PS-ingredient (PS-Ingr.) or β-sitosterol (β-sito) on (A) viability (MTT assay), (B) cell cycle and (C) apoptosis of HeLa cells. Data are expressed as mean ± SD; MTT assay: different lowercase letters (a–b) or different capital letters (A–D) indicate statistically significant differences (p < .05) among the different treatments after 24 or 48 h of incubation, respectively; # Denotes statistically significant differences between 24 and 48 h for a same treatment. Cell cycle assay: different lowercase letters (a–d) indicate statistical significant differences (p < .05) among the different treatments after 48 h of incubation; Apoptosis assay: percentage of Annexin V/propidium iodide (PI) double-stained cells presented are representative images of three experiments in triplicate.
due to the fact that HT-29 and Caco-2 cells have mutated the tumour suppressor protein p53, while HCT116 cells have functional p53 (p53 wild type) (Ahmed et al. 2013). Accordingly, tumours with mutated p53 can have a higher proportion of proliferating cells, and may be more metastatic than similar tumours with wild-type p53 (Brown and Wouters 1999).

In agreement with the present work, several studies have demonstrated that the antiproliferative effects of PS may be due to their ability to modulate cell cycle progression and apoptosis; however, variability of response was reported. In coincidence with our findings, Choi et al. (2003) observed in HCT116 cells that β-sitosterol (2.5–20 μM) induces apoptosis by increasing the sub-G1 cell population (3.8–16.5%) after 48 h, this effect being more accentuated from 7.5 μM. In this regard, Cilla et al. (2015) also observed apoptosis of Caco-2 cells through an increase in the number of cells in sub-G1 phase (145%) with exposure to standard PS mix at serum concentrations (13.2 μM), while β-sitosterol standard (12 μM) produced no significant change in this phase – though arrest in G2/M (16%) was recorded at 24 h. In contrast, other authors observed different behaviour of the PS upon cell cycle.Montserrat-de la Paz et al. (2015), using only β-sitosterol at 100 μM, have reported statistically significant irreversible arrest in phase G0/G1 of the cell cycle in HT-29 cells – this effect not being significant for β-sitosterol at 50 μM. In this same regard, López-García et al. (2017b) showed that standard PS mix (132 μM) and β-sitosterol (115 μM) also increase the number of cells in phase G0/G1. This suggests that β-sitosterol or PS mix modulates Caco-2 cell growth by blocking phase G0/G1 at high concentrations (100–132 μM).

Other mechanisms involved in the inhibitory effect of PS upon colon cancer cells have been reported, mainly associated with the apoptosis pathway, as in our own study. In the cell line used in the present work (HCT116), Choi et al. (2003) previously showed that the apoptosis mechanism of β-sitosterol (2.5–20 μM) could be mediated by caspase-3 and caspase-9 activation, and can be associated with decreased expression of the anti-apoptotic Bcl-2 protein, with a concomitant increase in pro-apoptotic Bax protein as well as with cytochrome c release from the mitochondria into the cytosol (Choi et al. 2003). In HT-29 cells, β-sitosterol (16 μM) was found to alter membrane lipids – suggesting that the inhibition of cell growth could be due to activation of the sphingomyelin cycle (involved in physiological parameters of apoptosis and cell growth), increasing ceramide production (50%) (Awad et al. 1996, 1998). Moreover, β-sitosterol at a higher concentration (100 μM), upregulated LXR-α and LXR-β gene expression (nuclear factors crucial for colon cancer progression) (Montserrat-de la Paz et al. 2015). On the other hand, PS antiproliferative activity against Caco-2 cells has been reported mediated by the mitochondrial pathway of apoptosis, secondary to an increase in cytosolic Ca2+ and oxidative stress (Cilla et al. 2015), or through necrotic cell death (López-García et al. 2017b). In this context, it has been suggested by López-García et al. (2017b) that PS exhibit a biphasic effect in Caco-2 cells, activating different molecular pathways depending on the concentration used, as observed on establishing comparisons with the results obtained in a previous report (Cilla et al. 2015). It seems that apoptosis is the main pathway implicated in cell death at low concentrations, while at high concentrations the apoptotic pathway may be suppressed, and cell death through necrosis prevails (López-García et al. 2017b).

The cell line involved in this study (HCT116) has also been used to evaluate the antiproliferative effect of PS isolated from plant extracts. In this regard, it has been reported that β-sitosterol and/or a mixture of PS (campesterol, stigmasterol and β-sitosterol) extracted from Pereskia bleo or Curcuma zedoaria does not display cytotoxic actions (IC50 > 100 μg/ml) (Malek et al. 2009; Rahman et al. 2013).

Cervical cancer cells

To the best of our knowledge, only one study (Cheng et al. 2015) has shown β-sitosterol (20 μM) present in Pinellias tuber to exert an antiproliferative effect (40%) upon HeLa cells at 24 h. A comparatively lesser antiproliferative effect was observed in our study (20.5%) for β-sitosterol (13 μM) at 24 h – the effect being similar (44.8%) at 48 h. Cheng et al. (2015) demonstrated that the β-sitosterol antiproliferative effect was due to reduction of the expression of proliferating cell nuclear antigen (PCNA), since β-sitosterol probably inhibits DNA synthesis in cells of this kind. Alterations in cell morphology were also observed (loss of cell surface microvilli, increased electron density of the cell membrane, and decreased organelle presence), suggesting that these cells gradually lose...
their malignant tumour characteristics upon treatment with β-sitosterol. The authors concluded that elevated levels of p53 mRNA (2.9-fold) and reduced levels of HPVE6 viral oncogenes (1.6-fold) observed upon treatment with β-sitosterol, could explain the anticancer activity against HeLa cells.

Most of the PS antiproliferative studies have been carried out with PS isolated from plant extracts upon HeLa cells. Han et al. (2013) observed that β-sitosterol isolated from Benicasa hispida at different concentrations (2.5–50 μM) did not show cytotoxic activity (inhibitory rate <35%) at 24 h. This behaviour is in agreement with our own work, since at 24 h, β-sitosterol at 26 and 52 μM showed no statistically significant (p < .05) antiproliferative effect, and β-sitosterol at 13 μM only produced 20.5% inhibition of cell growth. Several studies (Block et al. 2004; Csupor-Loffler et al. 2011) have observed that mixtures of β-sitosterol and stigmasterol isolated from Croton zambesicus or Conyza Canadensis, respectively, exerted no cytotoxic effect (IC₅₀ > 30 μg/ml) at 72 h. Moreover, with shorter exposure times (48 h), Hamdan et al. (2011) reported that a mixture of β-sitosterol and stigmasterol isolated from Citrus jambhiri Lush exhibited an IC₅₀ at 114.2 μM. The authors suggested that the cytotoxic activity could be due to the lipophilic character of these compounds, since they may interact with the lipophilic side chains of phospholipids or cholesterol, and in turn affect membrane fluidity and disrupt cell membrane function by modifying the three-dimensional conformation of membrane proteins. These observations are in agreement with our own results, since PS-ingredient (containing β-sitosterol and stigmasterol) at the concentrations tested (13, 26 and 52 μM) did not reach 50% proliferation inhibition in HeLa cells after 24 or 48 h.

**Conclusions**

The antiproliferative effect of β-sitosterol or PS-ingredient upon MCF-7, HTC116 and HeLa cells was generally evident at the concentration of 13 μM – this being compatible with physiological serum levels after the regular intake of a beverage containing these compounds. A possible biphasic/hormetic effect (with a greater response at lower concentrations versus higher concentrations) and/or a threshold of optimal action at the lowest assayed concentrations cannot be ruled out. Anticarcinogenic activity was observed through the induction of DNA fragmentation and apoptosis, with no clear time- and dose-dependent effect. With all the caution called for in drawing conclusions from *in vitro* assays, our results suggest that the PS-ingredient used for enrichment of functional beverages and its main PS (β-sitosterol), may be regarded as natural anticarcinogenic compounds against breast, colon and cervical cancer in these preclinical models, though further research is warranted. These observations may be complementary to the decrease in risk of cardiovascular disease and osteoporosis previously reported after the intake of a PS-enriched functional beverage containing the PS-ingredient assayed in this study; moreover, they should be taken into account by the food industry for using this PS-ingredient in the development of PS-enriched functional beverages, with a view to maximising their functionality.

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