The sterols isolated from evening primrose oil inhibit human colon adenocarcinoma cell proliferation and induce cell cycle arrest through upregulation of LXR.

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Short Communication

Conflict of interest: We declare that we do not have any financial or other relationships that might lead to a conflict of interest.

Abstract

Evening Primrose oil (EPO) is widely used as a dietary supplement from which beneficial effects have been reported in rheumatic and arthritic conditions, atopic dermatitis, psoriasis, premenstrual and menopausal syndrome, and diabetic neuropathy. The aim of this study was to determine whether phytosterols isolated from evening primrose oil (PS-EPO), and its main components β-sitosterol and campesterol, affect proliferation, cell death, and the cell cycle of human colon adenocarcinoma (HT-29) cells. PS-EPO were a potent antiproliferative agents in a dose- and time- dependent manner, with an IC_{50} of 62.9 μg/mL after 48 h, lower than β-sitosterol and campesterol (79.0 μM and 71.6 μM respectively). Flow cytometry showed that PS-EPO exerted a stimulatory effect on apoptosis and necrosis, increasing the number of cells in G0/G1 phase. PS-EPO produced a significant upregulation in liver X receptor (LXR) gene expression that may be one of the principal mechanisms of the tumour shrinkage by PS-EPO.

Keywords: Sterols; β-sitosterol; campesterol; evening primrose oil; Human colon adenocarcinoma cancer cells; HT-29.

1. Introduction

Colon cancer represents almost 10% of all tumours and is the third most common cancer in men in modern countries (after lung and prostate cancers) and the second in women (after breast cancer), with approximately 1 million new cases each year worldwide. The fact that only 5-10% of all cancer cases are due to genetic factor and that the remaining 90-95% are due to lifestyle factors such as diet, infections, and environmental pollutants provides major opportunities for preventing cancer (Jaramillo et al., 2010; Ferlay et al., 2010). The association between colon cancer, several selected food, and nutrient factors is clear. Epidemiological studies have associated a diet high in vegetables, fruits, cereals and seeds with a lower risk of colon cancer. In particular, food constituents such as vegetable oils and their components have been reported to reduce colon cancer risk experimentally (Vizzotto et al., 2014; Luna Vital et al., 2014; Cardeno et al., 2013a; 2013b).

Oil obtained from the seeds of evening primrose (*Oenothera biennis* L., Onagraceae) has attracted much interest due to its high content of polyunsaturated fatty acids, in particular, γ-linolenic acid (18:3n-6) and the beneficial effects of evening primrose oil (EPO) has been attributed to its fatty component. In contrast, little effort has been expended to characterize the beneficial biological effects of non-triacylglycerol constituents of EPO (Montserrat-de la Paz et al., 2012; 2014a). The unsaponifiable portion, about

1.5-2% of the oil, contains elevated amounts of sterols, 4-methylsterols, tocopherols, triterpene alcohols, hydrocarbons and alcohols (Montserrat-de la Paz et al., 2014b).

Phytosterols (PS) are the counterparts of cholesterol in animals. *In vitro* and animal studies suggest that PS offer protection from the most common cancers in developed countries, including colon, prostate, and breast (Awad et al., 2000). However, the exact mechanism by which dietary phytosterols offer this protection is not fully understood. Dietary consumption of PS is lower in developed countries (80 mg/day) as compared to developing countries (400 mg/day). It seems that the incidence and/or the death rate from these cancers that is minimal in developing countries may be due to the consumption of PS (Jayaprakasha et al., 2010).

Furthermore, several studies have reported that phytosterol and, in particular, β-sitosterol supplementation replaces cholesterol and reduces growth and survival signals routed through caveolar rafts in membranes of several cancer cell lines (Lopez et al., 2014; Hac-Wydro, 2010). Among the many mechanisms by which phytosterols exhibit anti-cancer activity *in vitro* are the modulation of ceramide metabolism, liver X receptor activation, cell cycle progression, and apoptosis (Bradford & Awad, 2010).

Taken this background into account, we designed the present study to investigate the effects of phytosterols isolated from evening primrose oil (PS-EPO) on HT-29 human colon adenocarcinoma cell proliferation, cell cycle, and apoptosis as well as to elucidate the underlying anticancer molecular mechanism involved, in particular, the role of liver X receptor (LXR).

2. Material and Methods

2.1. Materials

All cell culture reagents were purshed from Gibco (Madrid, Spain). The rest reagents were purchased from Sigma Aldrich Chem. (Madrid, Spain).

2.2. Isolation of phytosterols

The sterols from evening primrose oil were isolated and analyzed as described by Montserrat-de la Paz (2012).

2.3. Cell Culture and treatment

The human colon adenocarcinoma grade II cell line (HT-29) was obtained from the European Collection of Cell Cultures and was manteined in McKoy's 5A medium supplemented with 10% of foetal calf serum, in presence of 100 mg/mL streptomycin and 100 U/mL penicillin at 37ºC and 5% CO_2 atmosphere. The cells were kept subconfluent at a density of 5 x 10^5 cells/mL. Stock solutions, 10 mg/mL) of PS-EPO and 10 mM of β-sitosterol and campesterol, were prepared in DMSO and diluted to desired concentration directly in the culture medium. The final concentration of dimethyl sulphoxide (DMSO) in the culture medium did not significantly influence cell response. The quantities of the compounds are expressed as their final concentration in the culture medium.

2.4. Antiproliferative assay

Cells were seeded at a density of 5000 cells per well in 96-well plates. The plates were incubated for 24 and 48 h. 5-Fluorouracile at 5 μM (5-FU) was used as a positive control. Cell viability was asseyed base don the ability of live cells

to reduce MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). The concentration of formazan was measured spectrophotometrically at 490 nm.

2.5. Cell Cycle Analysis by Flow Cytometry

Cells were grown in 24-well plates at 37 °C under 5% CO_2 and incubated with the treatments for 24 and 48 h. After incubation, the cell pellet was resuspended in 200 μL of 70%ice-cold ethanol and 200 μL of PBS and stored at 80 °C until further use. For use in flow cytometry experiments, the cell pellet was suspended in 0.5 mL of staining reagent (50 μg/mL PI, 50 U/mL RNase, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and PBS) and incubated for 30 min at 37 °C in the dark. DNA fluorescence was measured using a Becton Dickinson (BD) FACScanto II flow cytometer with an excitation wavelength of 488 nm and emission wavelength of 585 nm. Pulse width area signals were used to discriminate between G2 cells and cell doublets. The data were analyzed using FACSDiva Software (BD). The relative distribution of 10000 events for each sample was analyzed for background aggregates and debris, an indicator of apoptosis as well as G0/G1, S, and G2/M phases of the cell cycle.

2.6. Assessment of Apoptosis and Necrosis

Apoptosis and necrosis were assessed using a Vybrant Apoptosis Assay kit # 2 (Molecular Probes, Barcelona, Spain) according to the manufacturer's instructions. Apoptotic cells were labeled with annexin V conjugated to green-fluorescent Alexa Fluor 488 dye, and necrotic cells were labeled with red-fluorescent PI. These populations were analyzed using a BD FACScanto II flow

cytometer with an excitation wavelength of 488 nm and a 530 nm filter for the detection of Alexa Fluor 488 and a 585 nm filter for PI detection. The data were analyzed using FACSDiva Software (BD). At least 10000 events for each sample were analyzed and gated according to light scattering properties.

2.7. RNA isolation and qRT-PCR analysis

Total RNA was also extracted from cells by using Trisure Reagent (Bioline), as instructed by the manufacturer. RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA (1 µg) was subjected to reverse transcription (iScript, Biorad) according to the manufacturers' protocol. An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (BioRad, CA, USA) containing the primer pairs for liver x receptor α (LXRα) gene, forward, *gaagaaactgaagcggcaaga*; reverse, *actcgaagccggtcagaaaa*; liver x receptor β (LXRβ) gene, forward, *tgcctggtttcctgcagct;* reverse, *agatgttgatggcgatgagca;* or for β-Actin, forward, *cgcaaagacctgtatgccaa*; reverse, *cacacagagtacttgcgctc* as housekeeping gene. All amplification reactions were performed in triplicate, and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (β-Actin) gene content and expressed as percentage of controls.

2.8. Statistical Analysis

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M.). Data were evaluated with Graph Pad Prism® Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Dunnett's multiple comparisons test as post hoc test. P values less than 0.05 were considered statistically significant.

3. Results and discussion

Sterols were isolated from the EPO unsaponifiable matter and represented a 49.40% of this fraction and almost a 1% from EPO (0.98%). This high proportion shows that EPO is one of the richest natural products in phytosterols versus others vegetal oils frequently used in the diet, like those coming from corn (0.95%) (Ostlund et al., 2002) or from sunflower (0.73%). Even the famous olive oil only has a 0.17% of phytosterols (Weihrauch & Gadner, 1978).

The identification of the different phytosterols was made using GC and GC-MS and comparing their RR_t values and MS fragments with literature values. Chromatographic analyses are shown in the **Fig. 1.** We consider very significant the proportion of campesterol (9.07 %) present in EPO as in other oils, like olive oil where the content reaches about 4 %. Since this compound has an advantage as a functional component, as its bioavailability in the body is 20 % compared with 7 % for sitosterol (Ostlund et al., 2002; Duan et al., 2004).

HT-29 cells were treated with diferent concentrations (0-100 µg/mL) of PS-EPO and (0-100 µM) of β-sitosterol and campesterol for 24 and 48 h. Cell viability in the presence of DMSO control alone was not affected. However, HT-29 cells were sensitive to treatments in a dose- and time-dependent manner (**Fig. 2A and 2B**). The IC_{50} values of PS-EPO for HT-29 cells were 93.50 µg/mL and 62.90 µg/mL after 24 and 48 h of incubation, respectively. These IC_{50} were lower than β-sitosterol-IC_{50} and campesterol-IC_{50}. Alterations of the intrinsic cell membrane asymmetry characteristic of apoptosis were detected by flow cytometry after 24 and 48 h of incubation with the phytosterol extract on the basis of annexin V binding to phosphatidylserine exposed in the outer leaflet of cell membrane. There was a statistically significant ($p < 0.001$), dose-dependent and time-dependent increase in annexin V-FITC conjugate fluorescence intensity (up to 2-fold vs control) (**Fig. 2C**), particularly after 100 µg/mL of PS-EPO incubation. We observed a high concentration of secondary necrosis (**Fig. 2D**), after 24 and 48h incubation with PS-EPO what is due to a earlier apoptosis induction that might be visible after shorter incubation time. Interestingly, PS-EPO at doses of up to 100 µg/mL was shown to arrest HT-29 cells preferentially at G0/G1 cell cycle phase after 48h (**Fig. 2E** and **2F**) when compared with the controls.

The extract did not cause a considerable increase in hypo-diploid peak in HT-29 cells after PS-EPSO at 100µg/mL (**Fig. 2E**). However, after 24h of incubation there was a slight, dose-dependent increase in the G2/M peak accompanied by a statistically significant decrease in the S peak, which could result from a G0/G1 cell cycle arrest (**Fig. 2F**). After an additional 24 h, the amount of apoptotic cells increased to a certain extent, which might be a

consequence of the putative G0/G1 block. These results also indicate that PS-EPO induced apoptosis and necrosis in HT-29 cells is likely to involve modulation of cell cycle progression.

To obtain further insights into the PS-EPO molecular mechanisms of action related to its antiproliferative effect, we evaluated the gene expression of different nuclear factors that are crucial in the progress of colon cancer, such as the activation of liver X receptors (LXRs). Strong evidences exist for such a protective role in prostate carcinoma, breast cancer cells, and colon carcinogenesis (Bradford & Awad, 2010). As shown, LXR-α (**Fig. 3A**) and LXR-β (**Fig. 3B**) gene expression was upregulated significantly after PS-EPO, β-sitosterol and campesterol treatments for 48 h. There exists a general recognition of the fact that LXR-α gene when activated in cancerous cells of diverse origin results in the regulation of genes coding for Bcl-2, AATF, and Par-4 in a fashion, forcing these cells to enter into the state of apoptosis. In this context the present study was addressed to understand the role of phytosterols from evening primrose oil on LXR-α gene modulation and its consequence on reprogramming of cancer cells into a state of apoptosis leaving the normal cells unaffected. The results of this study demonstrated that PS-EPO are involved in phytosterol-activated LXR serving a cancer protective role of evening primrose oil.

4. Conclusions

On the basis of this study we propose that in near future evening primrose oil phytosterols as a LXR-α agonist that may definitely find its use in

the therapeutic interventions directed towards the treatment of cancer. This is the first study demonstrating that PS-EPO are involved in phytosterol-activated LXR serving a cancer protective role.

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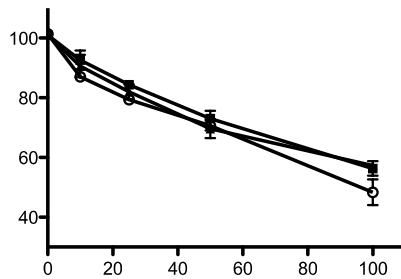
Figure 1
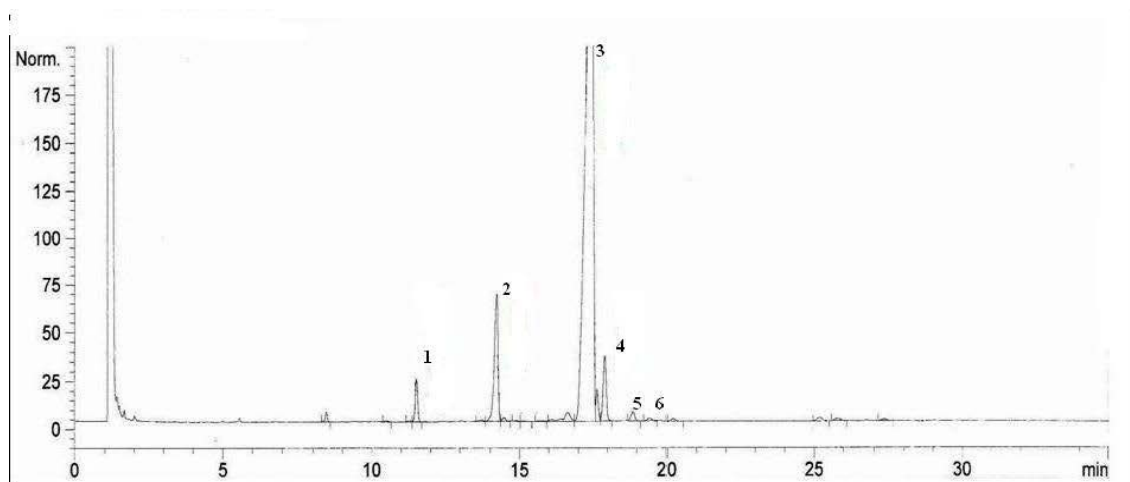
Fig. 1. Gas chromatogram of the sterols fraction from Evening Primrose Oil. **1.** α-Colestanol.

2. Campesterol (9.07%). **3.** β-Sitosterol (85.34%). **4.** Sitostanol (1.42%). **5.** Δ^5-Avenasterol (3.96%). **6.** Δ^7-Avenasterol (0.20%).

Figure 2

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Fig. 2 - Effect of PS-EPO and its main components on proliferation and cell cycle in HT-29 cells. Effect of PS-EPO (0-100 µg/mL) and β-sitosterol and Campesterol (0-100 µM) on viability of HT-29 assessed by MTT assay **(A)** for 24 h and **(B)** for 48 h. Effect os PS-EPO on apoptosis and necrosis of HT-29 cells assessed by FACS analysis. HT-29 cells were treated with DMSO, 5 µM 5-fluorouracyl (5-FU), PS-EPO (50 and 100 µg/mL) or β-sitosterol and Campesterol (50 and 100 µM) for 24 and 48 h. Cells were stained with annexin V **(C)** or propidium iodide **(D)**. Effect of PS-EPO on cell cycle distribution of HT-29 cells assessed as described in Materials and Methods (**E** and **F**). The data represents the average median of at least three independent

experiments (by triplicate) ± SD.   * $P < 0{,}05$, ** $P < 0{,}01$ and *** $P < 0{,}001$ vs control (DMSO) were determined by one-way analysis of variance followed by Dunnett's test.

Figure 3
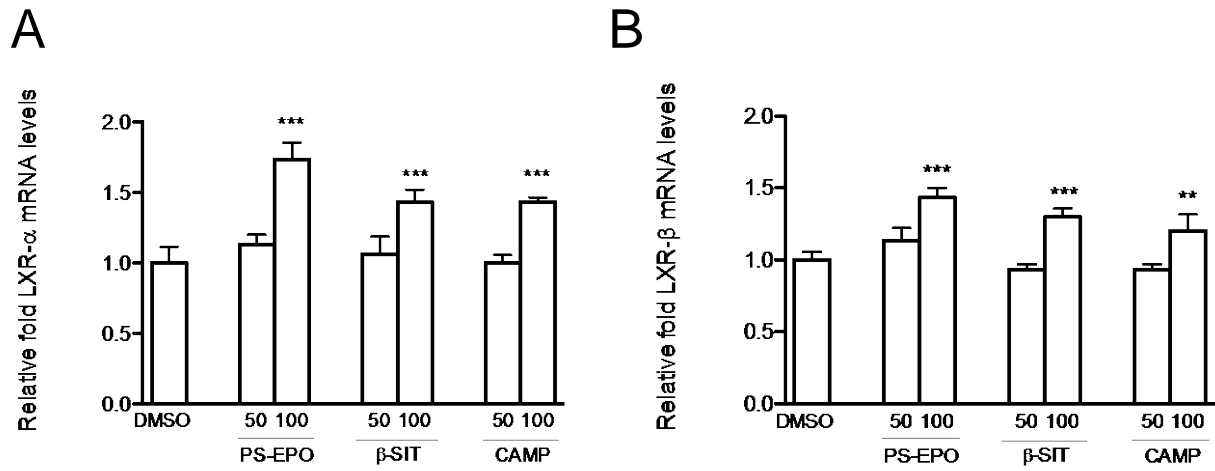
Fig 3 - Effect of PS-EPO ants its main components on LXR mRNA levels in HT-29 cells. Effect of PS-EPO (50 and 100 µg/mL) or β-sitosterol and Campesterol (50 and 100 µM) for 48 on LXR-α gene expression (**A**) and LXR-β gene expression (**B**) The data represents the average median of at least three independent experiments (by triplicate) ± SD. ** P < 0,01 and *** P < 0,001 vs control (DMSO) were determined by one-way analysis of variance followed by Dunnett's test.