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Saponins from edible spears of wild asparagus inhibit AKT, p70S6K, and ERK signalling, and induce apoptosis through G0/G1 cell cycle arrest in human colon cancer HCT-116 cells

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Abbreviations: **AKT**, RAC-alpha serine/threonine-protein kinase; **AMC**, 7-amino-4-methylcoumarin; **DMSO**, dimethyl sulfoxide; **dSAP**, digested saponin extract; **ERK**, extracellular signal–regulated kinase; **HT**, Huetor-Tajar; **HTSAP**, Huetor-Tajar asparagus saponin; **IC50,** half maximal inhibitory concentration; **KRAS**, Kirsten rat sarcoma viral oncogene homolog; **MS/MS**, tandem mass spectrometry; **mTOR**, mammalian target of rapamycin; **MTT**, methylthiazolyldiphenyl-tretrazolium bromide; **m/z**, mass to charge ratio; **p70S6K**, S6 kinase; **PARP-1**, poly(ADP-ribose) polymerase 1; **PIK3CA**, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha.

Abstract

The effects of steroidal saponins from edible spears of wild *triguero* Huetor-Tajar asparagus on some of the oncogenic molecular pathways that are affected in human colon cancer cells were investigated. Reverse-phase chromatography and a new HPLC–MS method were used to respectively isolate and analyse the composition of the steroidal saponins. They were resistant to simulated digestion and, when in contact with HTC-116 human colon carcinoma cells, interfered with extracellular signal–regulated kinase (ERK), S6 kinase (p70S6K, mTOR), and RAC-alpha serine/threonine-protein kinase (AKT) pathways by a downregulation of these proteins. The expressions of cyclins D, E, and A were also decreased, leading to G0/G1 cell cycle arrest. In addition, these steroidal saponins induced typical features of apoptosis by the promotion of caspase-3 activity, poly(ADP-ribose) polymerase 1 (PARP-1) cleavage, and DNA fragmentation. These results offer potential dietary intervention strategy against human colon cancer cells.

Keywords: Apoptosis, asparagus, cell cycle, colon cancer, human, saponins.

1. Introduction

Worldwide, there is a growing demand for high-quality, safe, health-promoting or disease-risk reducing foods. Therefore, the nutraceutical and therapeutic potential of dietary bioactive compounds is of increasing interest to both the scientific community and the food industry. Colon cancer represents almost 10% of all tumours. It is the third most common cancer in men in developed countries (after lung and prostate cancers) and the second in women (after breast cancer) with approximately 1 million new cases in the world each year. Only 5-10% of these cases are due to genetic factors, while more than 70% have their roots in the diet and other lifestyle factors (van Duijnhoven et al., 2009).

Previous studies have shown that saponins, which are naturally occurring compounds widely distributed in a variety of plants, exhibit anticancer activity by inducing cell cycle arrest and apoptosis, and by inhibiting proliferation in numerous types of human cancer cells (Bhardwaj et al., 2014; Liu & Li, 2014; Zhang et al., 2014). Saponins refer to a diverse family of secondary metabolites that contain a sugar moiety glycosidically linked to a hydrophobic aglycone. Legumes, especially soybeans (Omizu, Tsukamoto, Chettri, & Tamang, 2011), chickpeas (Ruiz et al., 1996), leeks (Fattorusso, Lanzotti, Taglialatela-Scafati, Di Rosa, & Ianaro, 2000), onions (Lanzotti, Romano, Lanzuise, Bonanomi, & Scala, 2012), and white asparagus (Lee, Yoo, & Patil, 2010) are the richest dietary source of saponins for humans. We have recently reported that a green wild asparagus (*triguero* Huetor-Tajar, HT, landrace), as a valuable ingredient of the Mediterranean diet, may also provide bioactive phytochemicals in relatively large amounts (Fuentes-Alventosa et al., 2008; Rodriguez et al., 2005) including

unusual steroidal saponins (Vazquez-Castilla et al., 2013a). The quantity of steroidal saponins was 10-100 times higher in the spears of *triguero* HT asparagus than those found in green asparagus commercial hybrids (Vazquez-Castilla et al., 2013b). It is noteworthy that chemoprevention of cancer by asparagus saponins is a property commonly explored in non-edible parts of the plant, such as roots (Liu et al., 2009), leaf extracts (Verma, Tripathi, & Das, 2014), fruits (Shao et al., 1999), and bottom-stems (Wang, Liu, Zhao, Zhang, & Pang, 2013). However, little is known about the anticancer potential activity of the saponin fraction from edible asparagus spears. Information pertaining to digestive stability of saponins from asparagus spears is also scarce. The aims of this study were to address the stability of saponins from spears of *triguero* HT asparagus during simulated digestion and to evaluate their anticancer effects and underlying mechanisms in human colon cancer HCT-116 cells.

2. Materials and Methods

2.1. Chemicals and reagents

Pepsin (EC 3.4.23.1) from porcine gastric mucosa, α-amylase (EC 3.2.1.1) and pancreatin from porcine pancreas, bile extract porcine, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protodioscin was purchased from Chromadex Chemical Co. (Barcelona, Spain). All cell culture reagents were purchased from Gibco (Madrid, Spain). Hoechst 33342 (H1399) was purchased from Molecular Probes (ThermoFisher Scientific, Madrid, Spain). Poly(ADP-ribose) polymerase 1 (PARP-1) antibody (7D3–6; 551024) was purchased from BD Pharmingen (San Jose, CA, USA), phospho-protein kinase

B (AKT, #4051), total-AKT (#2920), phospho-S6 kinase (p70S6, #9205), total-p70S6 (#9202) antibodies were purchased from Cell Signalling (Danvers, MA, USA). Phospho-extracellular signal–regulated kinase (ERK, M8159), total-ERK (M3807), and β-tubulin (T7816) antibodies were purchased from Sigma Chemical Co. Cyclin D (sc-8396), cyclin A (sc-751), and cyclin E (sc-198) were purchased from Santa Cruz Biotechnology (Texas, TX, USA).

2.2. Taxonomic identification and authenticity of *triguero* Huetor-Tajar asparagus

The *triguero* HT asparagus is also known as “Morado de Huetor” and the only tetraploid asparagus cultivar, together with the Italian “Violetto d’Albenga”, in Europe (Moreno et al., 2006). Previous studies on strain identification and phylogeny have suggested that the *triguero* HT asparagus is close to *Asparagus officinalis* and *A. maritimus* (Moreno et al., 2008a, 2008b). Since 2000, the European Commission registered the *triguero* HT asparagus as Protected Geographical Indication in the European Union (European Commission, 2000). Only fresh spears of the *triguero* asparagus harvested from HT landrace, Granada (Spain), were used in the present study.

2.3. Preparation of saponin extract

Spears from the *triguero* HT asparagus were extracted with 70% acetone as previously described (Vazquez-Castilla et al., 2013b). Briefly, the extract was loaded onto a column of Amberlite XAD-16 (ratio 170 mL resin/kg of fresh asparagus spears). The column was washed with water followed by 20%, 40%, and 96% of ethanol, using a ratio of 1/4 (v/v) versus the sample loaded. The

saponins were eluted with 40% of ethanol.

2.4. *In vitro* digestion of saponin extract

The mouth, the stomach, and the small intestine are compartments of the gastrointestinal tract that determine the bioaccessibility of any nutrient, including the saponins. It has been established the composition of digestive solutions that mimic the variety of digestive levels in the human physiology (Boyer, Brown, & Liu, 2005). The detailed components of these digestive solutions are listed in the supplementary material (Table S1). Samples of saponin extract (5 mg) were first homogenized with 10 mL portions of saliva-like solution (including α-amylase) and incubated in a water-bath with agitation for 5 min at 37 °C (mouth-like digestion); the samples were then acidified to pH 2.0 with HCl before the addition of pepsin (200 mg in 5 mL of 0.1 M HCl) and incubated with agitation for 1 h at 37 ºC (gastric-like digestion); afterwards, the pH was increased up to 5.5-6.0 with 1 M $NaHCO_3$ and pancreatin (4 mg/mL), and bile extract (25 mg/mL) were added, while the mixture was finally incubated with agitation for 2 h at 37 ºC (small intestinal-like digestion). Aliquots from five independent replicates were taken at the middle and end of each digestive-like process, rapidly cooled on ice and lyophilized. Controls without digestive enzymes were run in parallel. Only aliquots obtained after the completion of the small intestinal phase of digestion (named digested saponin extract, dSAP) were used for cell culture experiments.

2.5. Saponin analysis by HPLC–MS

The identification and quantification of saponins were carried out in extracts

subjected or not subjected to *in vitro* digestion applying a HPLC-MS based method (Waters 600; Waters, Milford, MA, USA) fitted with a C-18 chromatography reverse-phase column (Mediterranean Sea18, 5-µm particles, 4.4 x 250 mm) supplied by Teknokroma (Barcelona, Spain). Solvents that constituted the mobile phase were acidified aqueous (A) and acidified acetonitrile (B) with 0.1% formic acid. The separation was achieved using gradient elution of 0-30 min, 20% B; 30-60 min, linear gradient to 30% B; 60 to 70 min linear gradient to 100% B and 70-80 min, linear gradient 20% B. The flow rate was set at 1 mL/min and the injection volume was 20 µL. The HPLC was interfaced to a MS quadrupole mass analyser (ZMD4, Waters) via an electrospray ionization (ESI) source operated in positive (capillary voltage at 50 eV) and negative (capillary voltage at 50 and 100 eV) modes; the cone voltage and collision energy were set at 3 kV and 12 V, respectively. The source and desolvation temperatures were 120 and 200 °C, respectively. The flow rate to the ESI source was decreased up to 200 µL/min by a splitter.

2.6. Cell culture

The human colon adenocarcinoma cell line HCT-116 was obtained from the ATCC (#CCL 247; Bethesda, MD, USA). Cells were grown at 37 ºC with 5% CO_2 and 90% relative humidity in McCoy's 5A medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

2.7. Cell viability assay

Cell viability was assayed based on the ability of live cells to reduce MTT

(Jaramillo et al., 2010). HCT-116 cells were cultured in 96-well plates at a density of 10^4 cells/well in 200 µL of medium. The cells were grown to 70-80% confluence, and then they were treated with vehicle (DMSO, <0.1%) or dSAP at indicated concentrations for 12, 24, and 48 h before adding the MTT solution for 3 h at 37 ºC. All MTT assays were carried out in three separate experiments, each one including five replicates. The osmolality of culture media supplemented with DMSO or dSAP was controlled with a Knauer semi-micro osmometer (Knauer and Co., Berlin, Germany).

2.8. Western blot analysis

Total cellular proteins, extracted from HCT-116 cells at different experimental conditions, were examined by western blot analysis as previously described (Lopez et al., 2013). Briefly, cells were washed once with PBS and gently lysed in RIPA buffer containing 1 mM Na_3VO_4, 1 mM NaF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatine, and 1 µM PMSF at 4 ºC with shaking for 30 min. Cell homogenates were then transferred in a 1.5 mL tube and centrifuged 10 min, 10000 x g at 4 ºC. Total proteins in the supernatant were collected, their concentration determined using the Bradford protein assay and stored at -80 ºC. Samples were subjected to SDS-PAGE, Western blot analysis and transferred onto nitrocellulose membrane (0.22 µm, Bio-Rad, Hercules, CA, USA). Membranes were immunoblotted with mouse anti-phospho-Akt, mouse anti-Akt, mouse anti-phospho-ERK1/2, mouse anti-ERK, mouse anti-PARP-1, rabbit anti-phospho-p70S6K, mouse anti-p70S6K, mouse anti-Cyclin D, rabbit anti-Cyclin A, rabbit anti-Cyclin E, and mouse anti-B-Tubulin 1 antibodies. Specific antigen-antibody complexes were detected with the SuperSignal West Pico

Chemiluminescent Substrate (ThermoFisher Scientific, Madrid, Spain). Protein loading equivalence was corrected in relation to the expression of β-tubulin.

2.9. Cell cycle analysis

Cell cycle distribution was analysed by flow cytometry as previously described (Jaramillo et al., 2010). Briefly, cells were washed three times with ice-cold PBS. The cell pellet was resuspended in 200 µL of 70% ice-cold ethanol and 200 µL of PBS, and stored at -80 ºC. For cell cycle analysis, cells were thawed, washed twice with PBS, and suspended in 0.5 mL of staining reagent (50 µg/mL PI, 50 U/mL RNase, 0.1 mM EDTA, 0.1% Triton X-100, PBS). After incubation for 30 min at 37 ºC in the dark, DNA fluorescence was measured using a Becton Dickinson (BD Biosciences, Madrid, Spain) 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. Data were analysed using FlowJo v5.7.2 software (Tree Star, Ashland, OR, USA). Approximately 10000 events per sample were analysed, and the relative distribution of cells in each phase (G0/G1, S, and G2/M) was displayed as histograms.

2.10. DNA condensation and fragmentation assays

Apoptosis was also determined by DNA condensation assay using Hoechst 33342 dye as previously described (Lopez et al., 2013). In addition, low-molecular-weight genomic DNA was evaluated following a previous method with some modifications (de Mello et al., 2015). Briefly, all the cells (including floating cells) were harvested and washed twice with PBS. Cells were treated with a

lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% sodium sarkosinate, 0.5 mg/mL RNAase A) for 1 h at 37 ºC, before the addition of 1 mg/mL proteinase K and an additional incubation for 3 h at 56 ºC. DNA was isolated by the phenol-chloroform-isoamyl alcohol method and dissolved in TE buffer (pH 7.4). A volume of the resulting solution containing 2 mg of DNA was loaded on 1.8% agarose gel. The isolated DNA was visualised by ethidium bromide staining under a UV lamp.

2.11. Caspase-3 activity assay

Caspase-3 activity was determined using a caspase-3 assay kit, Fluorimetric (CASP3F, Sigma-Aldrich) as previously described (Lopez et al., 2013). The cells were incubated with the substrate, acetyl Asp-Glu-Val-Asp 7-amino-4-methylcoumarin, and the fluorescence intensity of the resulting 7-amino-4-methylcoumarin (AMC) product was recorded at wavelengths of 360 and 460 nm for excitation and emission, respectively.

2.12. Statistical analysis

Data were analysed using IBM® SPSS® statistics v23.0 for Windows (IBM, Madrid, Spain). All quantitative data are represented as mean ± SD from triplicate experiments performed in parallel unless otherwise indicated. Mean values among treatment groups were compared by the ANOVA followed by Duncan's multiple comparison test. A level of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Characterization of saponins in extracts from edible spears of *triguero* Huetor-Tajar asparagus

The amount of saponins quantified in fresh wild asparagus was 4.6 mg/100 g of fresh spear. There were five major peaks of *triguero* HT asparagus saponins (HTSAP): HTSAP-1, HTSAP-2, HTSAP-12, HTSAP-6, and HTSAP-8 (Figs. 1A-1B). The tandem mass (MS/MS) spectrum showed that all of these saponins are derived from a furostan-type steroidal genin with a single bond between C5-C6 of the B ring; they have been described in previous reports (Vazquez-Castilla et al., 2013a, 2013b). The most abundant saponins HTSAP-1, HTSAP-12, and HTSAP-6 represented more than 90% of the total saponin content from edible spears of *triguero* HT asparagus (Table 1).

3.2. Steroidal saponins from edible spears of *triguero* Huetor-Tajar asparagus are stable to a simulated digestion

The exposure of steroidal saponins to sequential incubations with oral-, gastric-, and small intestinal-like fluids did not substantially affect their stability. Representative chromatograms of steroidal saponins in fractions from edible spears of *triguero* HT asparagus following different phases of simulated digestion are shown in the supplementary material (Figure S1). All of the steroidal saponins remained stable with a final recovery range of 87.5-94.2% (Table 2). Similar stability was observed for samples of steroidal saponin extracts subjected to fluids with salts but without enzymes (data not shown).

3.3. Steroidal saponins from edible spears of *triguero* Huetor-Tajar asparagus are cytotoxic to human colon cancer HCT-116 cells

Aliquots of saponin extracts obtained after the completion of simulated oral, gastric, and small intestinal digestion (dSAP) were used for successive experiments. HCT-116 cells were treated with different concentrations (0-250 µg/mL) of dSAP for 12, 24, and 48 h. dSAP was cytotoxic in a dose- and time-dependent manner ($p < 0.05$, Fig. 2). The concentration producing a 50% of cell death (IC50) of dSAP on HCT-116 cells was ~193 µg/mL for 24 h and ~76 µg/mL for 48 h. No further decrease in cell viability was detected at concentrations of dSAP above 200 µg/mL.

3.4. Steroidal saponins from edible spears of *triguero* Huetor-Tajar asparagus inhibit the phosphorylation of AKT, p70S6K, and ERK in human colon cancer HCT-116 cells

We performed the expression of AKT, p70S6K, and ERK by western blot in HCT-116 cells treated with dSAP at IC50 concentration for 48 h. The dSAP decreased the expression of these cell survival proteins in HCT-116 cells ($p < 0.05$, Figs. 3A-3D).

3.5. Steroidal saponins from edible spears of *triguero* Huetor-Tajar asparagus induce caspase-3 activity, PARP-1 cleavage, and DNA fragmentation in human colon cancer HCT-116 cells

Caspase-3 activity was significantly increased ($p < 0.05$) in HCT-116 cells treated with dSAP at IC50 concentration for 48 h (Fig. 4A). As expected, PARP-1 cleavage occurred in HCT-116 cells treated with dSAP (Figs. 4B-4C). After cellular DNA content analysis, we observed that dSAP induced apoptosis and DNA fragmentation ($p < 0.05$) of HCT-116 cells by means of hypodiploid DNA

content in the sub-G0/G1 cell population (Fig. 4D). These effects of dSAP on HCT-116 cells were confirmed by a characteristic DNA-ladder during DNA gel electrophoresis (Fig. 5A) and by pyknotic nuclei under fluorescence microscopy (Figs. 5B-5E).

3.6. Steroidal saponins from edible spears of *triguero* Huetor-Tajar asparagus induce cell cycle arrest by reducing the expression of cyclins D, A, and E in human colon cancer HCT-116 cells

We determined the cell cycle distribution in HCT-116 cells treated with dSAP at IC50 concentration for 48 h. The dSAP increased the number of HCT-116 cells in sub-G0/G1 and arrested HCT-116 cells in G0/G1 phase at the expense of those in G2/M phase ($p < 0.05$, Figs. 6A-6B). The western blot analysis of cyclins D, A, and E confirmed that dSAP decreased the expression of these cell-cycle stimulating proteins in HCT-116 cells ($p < 0.05$, Figs. 6C-6F).

4. Discussion

In this study, we have applied a new HPLC–MS method (Vazquez-Castilla et al., 2013b) to analyse the composition of saponins in the spears of *triguero* HT asparagus. This edible part of the plant was found to provide high amounts of furostanol steroidal saponins that differ from protodioscin, which is the most abundant of asparagus steroidal saponins (up to 85% of the total saponin concentration in white asparagus spears) (Fuentes-Alventosa et al., 2009; Lee, Yoo, & Patil, 2010). While protodioscin has a double bond between carbons 5 and 6 of the B ring in the six-ringed C_{27} skeleton, steroidal saponins from the spears of *triguero* HT asparagus have a single bond between C5 and C6. We

identified three major saponins derived from a furostan-type steroidal genin (HTSAP-1, 6, and 12) accounted for 90% of total saponins in the spears of *triguero* HT asparagus. Steroidal saponins from non-edible parts of asparagus (*A. racemosus, A. filicinus, A. officinalis L., A. oligoclonos*) have been reported to exhibit important pharmacological activities, including immunomodulation (Gautam et al., 2009; Pise, Rudra, & Upadhyay, 2015) and cytotoxicity (Bhutani, Paul, Fayad, & Linder, 2010; Kim et al., 2005; Liu et al., 2009; Wu et al., 2010; Zhou et al., 2007) on several human tumour cells. To our knowledge, this is the first report focused on anticancer effects of steroidal saponins from spears as an edible part of asparagus, which in this case is an autochthonous green asparagus variety of the type *triguero* from the south of Spain (HT landrace, Granada) (Vazquez-Castilla et al., 2013a). It is worth mentioning that the absorption of ingested saponins is very low (Liu et al., 2013; Okawara, Tokudome, Todo, Sugibayashi, & Hashimoto, 2014) and the stability of steroidal saponins to digestive processes is not fully understood; yet this is the first study addressing the stability of asparagus steroidal saponins during simulated oral, gastric, and small intestinal digestion. Steroidal saponins from the spears of *triguero* HT asparagus remained stable with greater than 90% recovery of initial amounts. Previous studies also reported a high stability of steroidal saponins in tea from ginseng root extracts by similar procedures of simulated digestion (Kim et al., 2014; Kim, Lee, Shin, Son, & Kim, 2009). Our observations on digestive resistance of steroidal saponins from the spears of *triguero* HT asparagus, together with the well-known low bioavailability of saponins, may suggest that these steroidal saponins remain in the gastrointestinal tract and are colon-accessible, where they may exert their biological effects. Thus, we observed an

inhibition of cell growth when human colon cancer HCT-116 cells were in contact with the artificial digest of steroidal saponins from the spears of *triguero* HT asparagus. Apart from protodioscin (Hu & Yao, 2002), our data are the first to demonstrate the cytotoxic activity of furostanol saponins from (an edible part of) asparagus on human colon cancer cells. Previous studies were mainly focused on cytotoxicity of spirostane-type steroidal saponins from non-edible parts of the asparagus (Bhutani et al., 2010; Kim et al., 2005; Liu et al., 2009; Wu et al., 2010; Zhou et al., 2007). Importantly, furostanol saponins have been shown to be less toxic for normal cells than spirostanol saponins (Lee et al., 2010).

Colonic epithelial cell line HCT-116 is considered a tissue culture model of apoptosis resistance in human colon cancer, in which genes Kirsten rat sarcoma viral oncogene homolog (KRAS, G13D) and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA, H1047R) are constitutively activated (Ahmed et al., 2013). Both oncogenes are downstream effectors of ERK, AKT, and mammalian target of rapamycin (mTOR) signalling pathways that promote cell growth, survival, and the transition from colon adenoma to carcinoma (Grady & Pritchard, 2014). Recent approaches to KRAS and PIK3CA mutant tumour treatment with ERK (Lamba et al., 2014) and PI3K/AKT/mTOR (Janku et al., 2013) inhibitors have stressed the need to expand resources available to suppress redundant signalling networks for cancer treatment. We found that steroidal saponins from the spears of *triguero* HT asparagus decreased the phosphorylated forms of ERK, AKT, and p70S6K (as a marker of mTOR activation) in HCT-116 cells. These findings in HCT-116 cells are in contrast to the increased phosphorylation of ERK by oleane-type

376 triterpenic saponins from aerial parts of *Glochidion eriocarpum* (Nhiem et al.,
377 2012), but are similar to the effects on AKT and mTOR phosphorylation by total
378 saponins from the roots of *Radix astragali* (Law, Auyeung, Chan, & Ko, 2012).
379 The steroidal saponin Aspafilioside B from the roots of *A. filicinus* has been
380 reported to increase the phosphorylation of ERK in human HepG2
381 (hepatocellular carcinoma) cells (Liu et al., 2015). Other non-asparagus
382 steroidal saponins have been shown to decrease the phosphorylation of ERK
383 and AKT in human HL-60 (promyelocytic leukemia), PC-3 (prostate cancer),
384 SNU-C5 (colorectal cancer), MCF-7 (breast cancer), MAD-231 (breast cancer),
385 and MCF Her2 (breast cancer) cells (Srinivasan et al., 2009; Thao et al., 2014).
386 Recent studies have also reported that the phosphorylation of protein kinases
387 p38 and JNK, mainly involved in apoptosis (Sui et al., 2014), are increased by
388 the dammarane-type triterpenic saponin Jujuboside B from *Zizyphus jujuba* in
389 HCT-116 cells and human gastric cancer AGS cells (Xu, Lee, Kang, & Kim,
390 2014). Therefore, the simultaneous suppression of ERK, AKT, and mTOR
391 signalling may be, at least in part, a possible mechanism by which steroidal
392 saponins from the spears of *triguero* HT asparagus induce a loss of viability in
393 HCT-116 cells. These observations underscore the selective efficacy of these
394 steroidal saponins of interfering any complementary and/or feedback interplay
395 between RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways (Gao, Shi, Xia,
396 Inagaki, & Tang, 2015). It is tempting to speculate that the amphiphilic character
397 of saponins could disturb the process of receptor tyrosine kinase activation at
398 the cytoplasmic membrane level (Lorent, Quetin-Leclercq, Mingeot-Leclercq,
399 2014), not excluding the possibility of dSAP inhibiting the receptor tyrosine
400 kinase domain (Sathishkumar et al., 2013). We further observed the entry of

401 cells in apoptosis by several means: enhancement of sub-G0/G1 cells,
402 caspase-3 activation, PARP-1 cleavage, and DNA fragmentation. In addition,
403 cyclins D, E, and A were downregulated and cells were arrested at G0/G1
404 phase of the cell cycle. Similar effects have been reported for non-asparagus
405 triterpenoid saponins in decreasing cyclin D expression and promoting cell cycle
406 arrest in the G0/G1 phase, accompanied by an inhibition of cell proliferation
407 and/or induction of apoptosis in HCT-116 cells (Du et al., 2012; Du et al., 2013;
408 Lee et al., 2015; Li et al., 2014; Zhang et al., 2015). Cyclin D is a pivotal
409 regulator of G0/G1 transition in cell cycle (Jayaraman & Jamil, 2014) and its
410 downregulation or inhibition has been previously attributed to STAT-dependent
411 mechanisms (Podolak, Galanty, & Sobolewska, 2010) or docking of bioactive
412 molecules into the active site of cyclins (Yang et al., 2015). It is likely that the
413 mechanisms involved in cell cycle modulation for G0/G1 arrest to induce
414 apoptosis are highly conserved in the hystotype of human colon cancer cells,
415 since asparagus steroidal saponins such as Asparanin A from the roots of *A.*
416 *officinalis* (Liu et al., 2009) and Aspafilioside B from the roots of *A. filicinus* (Liu
417 et al., 2015) exhibit cytotoxic effects by affecting a different profile expression of
418 cyclins and increasing caspase-3 activation and PARP cleavage, though
419 arresting the cell cycle at the G2/M phase in human HepG2 (hepatocellular
420 carcinoma) cells. We did not observe direct effects of dSAP on lysis in HCT-116
421 cells, which is in line with the low potency of steroidal saponins to induce
422 membrane destabilization with cytoskeleton integrity disassembly when
423 compared to triterpene saponins (Podolak, Galanty, & Sobolewska, 2010).
424

425 ## 5. Conclusions

In summary, our study suggests that steroidal saponins from edible spears of *triguero* HT asparagus are stable to simulated digestion. These saponins, when in contact with human colon cancer HCT-116 cells, block ERK, AKT, and p70S6 (mTOR) signalling pathways; arrest the cell cycle at G0/G1 phase by interfering the expression of cyclins D, E, and A; and induce cell death through the apoptotic pathway via caspase-3, leading to PARP-1 cleavage and DNA fragmentation. The potential relevance of these steroidal saponins on other human cancer cell types to translate into cancer care warrants further research.

Conflict of interest

The authors have declared no conflicts of interest.

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690 **FIGURE CAPTIONS**

691 **Fig. 1.** Mass spectrometric analysis of the steroidal saponin extract from edible
692 spears of *triguero* HT asparagus. 1: HT asparagus saponin (HTSAP)-1; 2:
693 HTSAP-2; 3: HTSAP-12, 4: HTSAP-6; 5: HTSAP-8 (A). Negative electrospray
694 ionization-MS spectra of the HTSAP-12 saponin (B). m/z, mass to charge ratio.
695
696 **Fig. 2.** Loss of cell viability of HCT-116 cells treated with digested saponin
697 extract (dSAP, 0-250 µg/mL) for 12, 24, and 48 h. Each point represents the
698 mean ± SD (n = 5). Two-way ANOVA analysis: Time effect: $p < 0.0001$; Dose
699 effect: $p < 0.0001$; and Time x Dose: $p < 0.0001$. *Statistical differences
700 between 24 and 48 h ($p < 0.05$). † Statistical differences between 12 and 24 h
701 ($p < 0.05$).
702
703 **Fig. 3.** Western blot analysis of total and phosphorylated form of AKT (A and B),
704 p70S6K (A and C), ERK (A and D), and β-tubulin (protein loading control) of
705 HCT-116 cells treated with digested saponin extract (dSAP, 76 µg/mL) for 48 h.
706 Untreated cells are referred as control. Each point represents the mean ± SD (n
707 = 3). *Statistical differences compared to control ($p < 0.05$).
708
709 **Fig. 4.** Caspase-3 activity (A), western blot of PARP-1 (B), quantification of
710 PARP-1 cleavage (C), and sub-G0/G1 analysis (D) of HCT-116 cells treated
711 with digested saponin extract (dSAP, 76 µg/mL) for 48 h. Untreated cells are
712 referred as control. Each point represents the mean ± SD (n = 3). *Statistical
713 differences compared to control ($p < 0.05$). AMC, 7-amino-4-methylcoumarin.
714

Fig. 5. Gel agarose electrophoresis (A) of DNA from HCT-116 cells treated with digested saponin extract (dSAP, 76 µg/mL) for 48 h (lane 2). DNA from untreated cells is shown in lane 1. Phase contrast (B and D) and hoescht 33342 staining (C and E) of HCT-116 cells treated with dSAP (76 µg/mL). Arrows indicate pyknotic nuclei.

Fig. 6. Cell cycle profile (A), cell cycle quantification (B), and western blot analysis of cyclin D, A, and E (C-F) in HCT-116 cells treated with digested saponin extract (dSAP, 76 µg/mL) for 48 h. Untreated cells are referred as control. Each point represents the mean ± SD (n = 3). *Statistical differences compared to control ($p < 0.05$).

Table 1. Saponin profile in edible spears of *triguero* HT asparagus.

Saponin	Rt (min)	Mw (Da)	mg/100 g FW
HTSAP-1	25.56	1052	1.25 ± 0.14
HTSAP-2	26.58	920	0.17 ± 0.03
HTSAP-12	31.08	1050	1.49 ± 0.13
HTSAP-6	32.01	1036	1.55 ± 0.16
HTSAP-8	33.40	904	0.14 ± 0.04

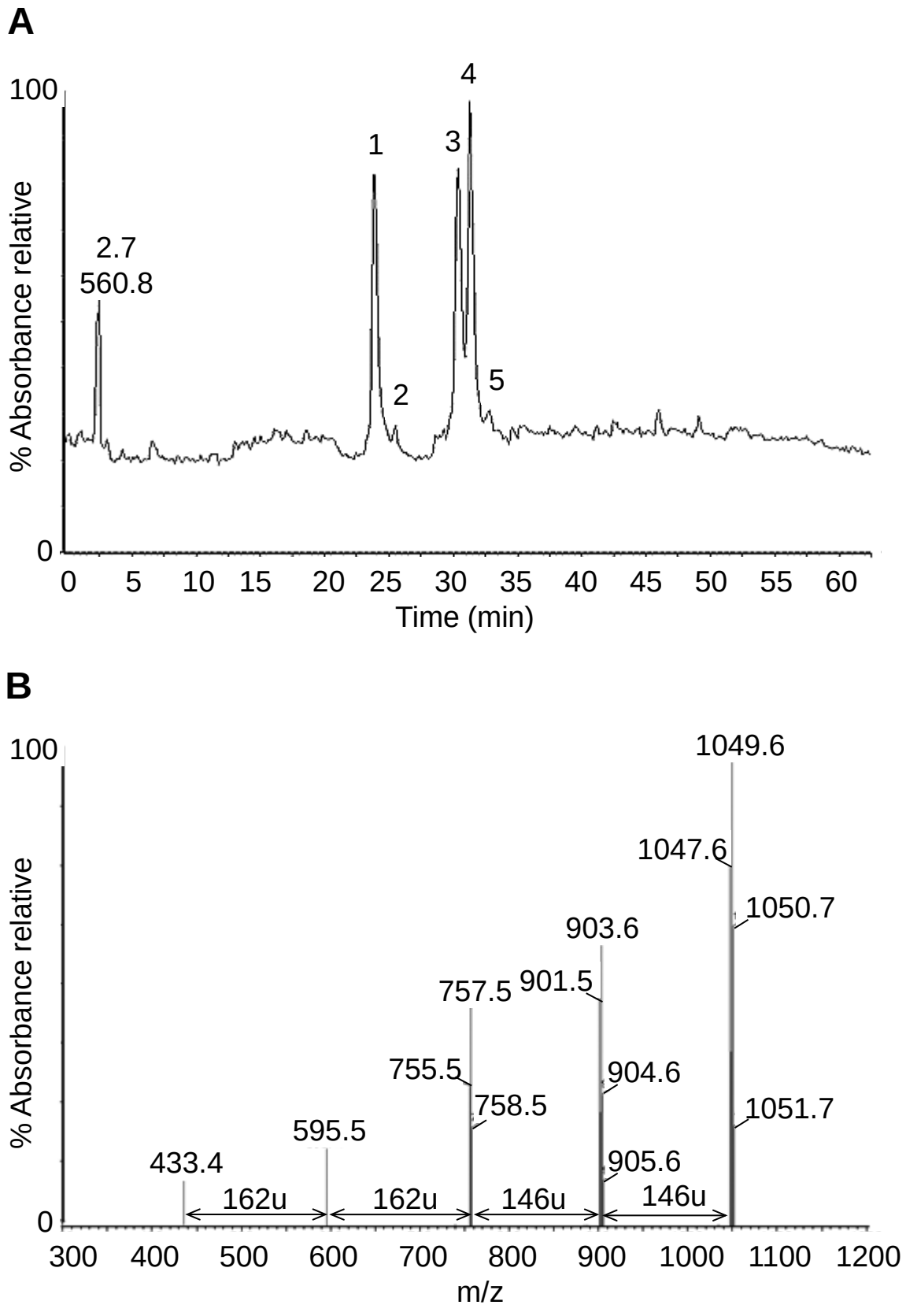
HT, Huetor-Tajar; HTSAP1-12, HT asparagus saponins; Rt, retention time; Mw, molecular weight; FW, fresh weight.

Table 2. Stability of saponins from edible spears of *triguero* HT asparagus during the *in vitro* digestion assay. Each saponin has been quantified (in μg/mL) at the end of each step of the model. The percentage of the original amount of each saponin is shown between parentheses.

Saponin	m/z (MS/MS)	Original	Mouth-like digestion	Gastric-like digestion	Small intestinal-like digestion	Total loss (%)
HTSAP-1	1051	198 ± 14	178 ± 7 (89.9%)	180 ± 8 (90.9%)	176 ± 8 (88.8%)	11.2%
HTSAP-2	919	28 ± 2	25 ± 1 (89.3%)	24 ± 1 (85.7%)	25 ± 1 (89.3%)	10.7%
HTSAP-12	1049	241 ± 13	222 ± 9 (92.1%)	226 ± 13 (93.8%)	227 ± 9 (94.2%)	5.8%
HTSAP-6	1035	250 ± 16	228 ± 10 (91.2%)	228 ± 10 (91.2%)	229 ± 9 (91.6%)	8.4%
HTSAP-8	903	24 ± 1	22 ± 1 (91.6%)	22 ± 1 (91.6%)	21 ± 1 (87.5%)	12.5%

HT, Huetor-Tajar; HTSAP1-12, HT asparagus saponins; MS/MS, tandem mass spectrometry; m/z, mass to charge ratio.

Fig. 1


A
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% Absorbance relative
0
2.7
560.8
1
2
3
4
5
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)
B
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% Absorbance relative
0
433.4
595.5
757.5
755.5
758.5
901.5
903.6
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905.6
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1047.6
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162u
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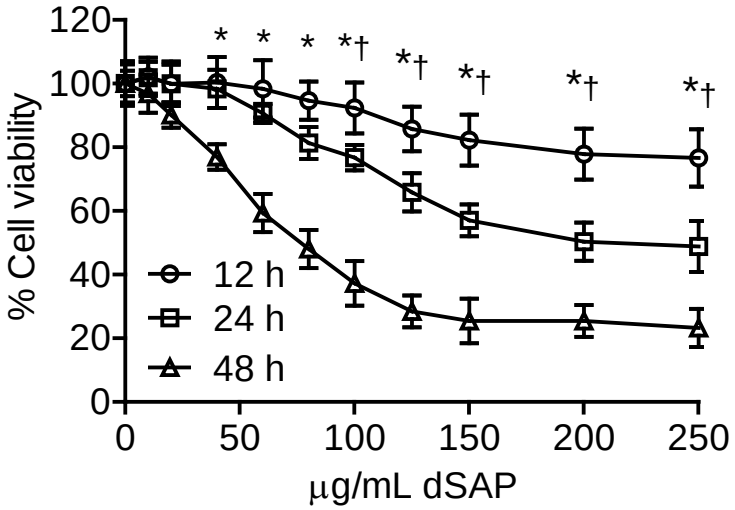
300 400 500 600 700 800 900 1000 1100 1200
m/z

Fig. 2


120
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80
60
40
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% Cell viability
0
50
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200
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μg/mL dSAP
12 h
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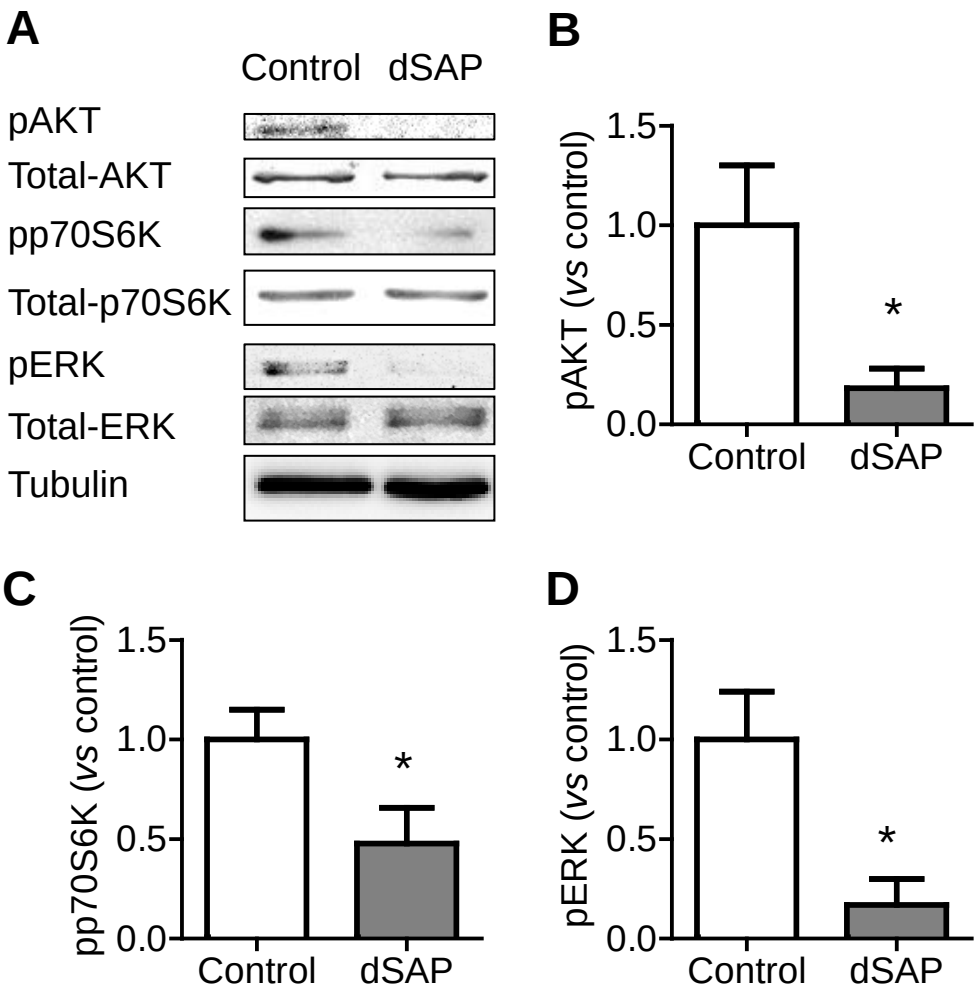
Fig. 3


A
Control
dSAP
pAKT
Total-AKT
pp70S6K
Total-p70S6K
pERK
Total-ERK
Tubulin
B
pAKT (vs control)
1.5
1.0
0.5
0.0
*
Control
dSAP
C
pp70S6K (vs control)
1.5
1.0
0.5
0.0
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Control
dSAP
D
pERK (vs control)
1.5
1.0
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*
Control
dSAP

Fig. 4

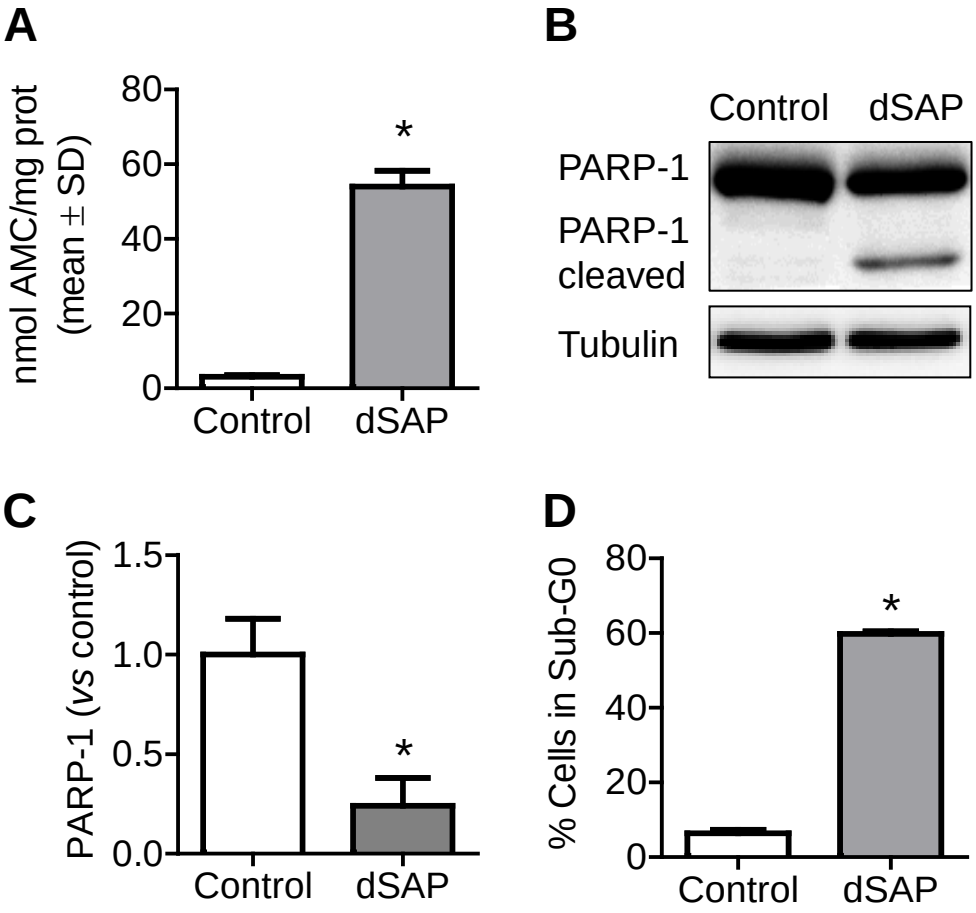

A
nmol AMC/mg prot
(mean ± SD)
80
60
40
20
0
*
Control
dSAP
B
Control
dSAP
PARP-1
PARP-1
cleaved
Tubulin
C
PARP-1 (vs control)
1.5
1.0
0.5
0.0
*
Control
dSAP
D
% Cells in Sub-G0
80
60
40
20
0
*
Control
dSAP

Fig. 5

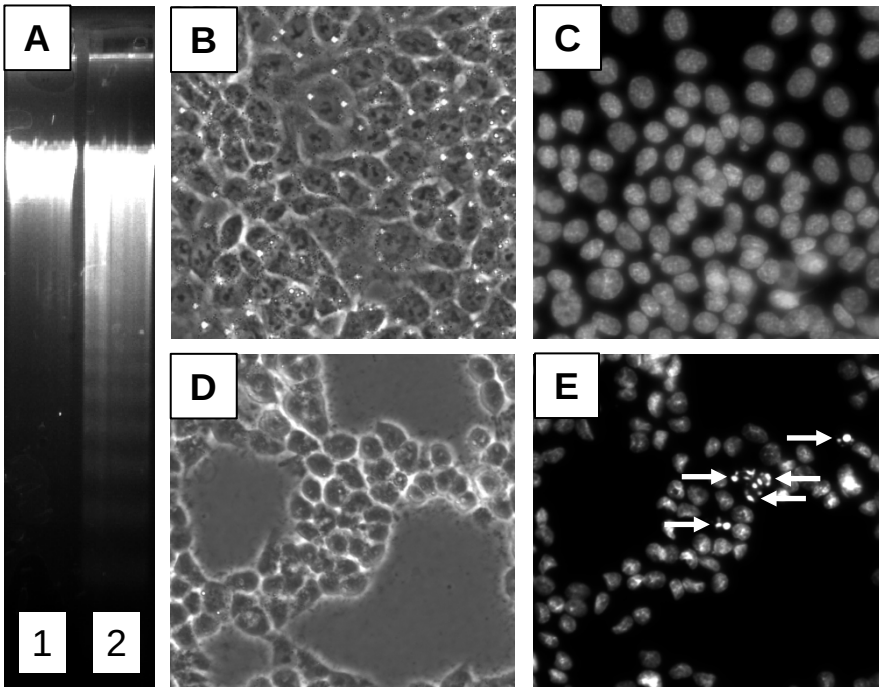
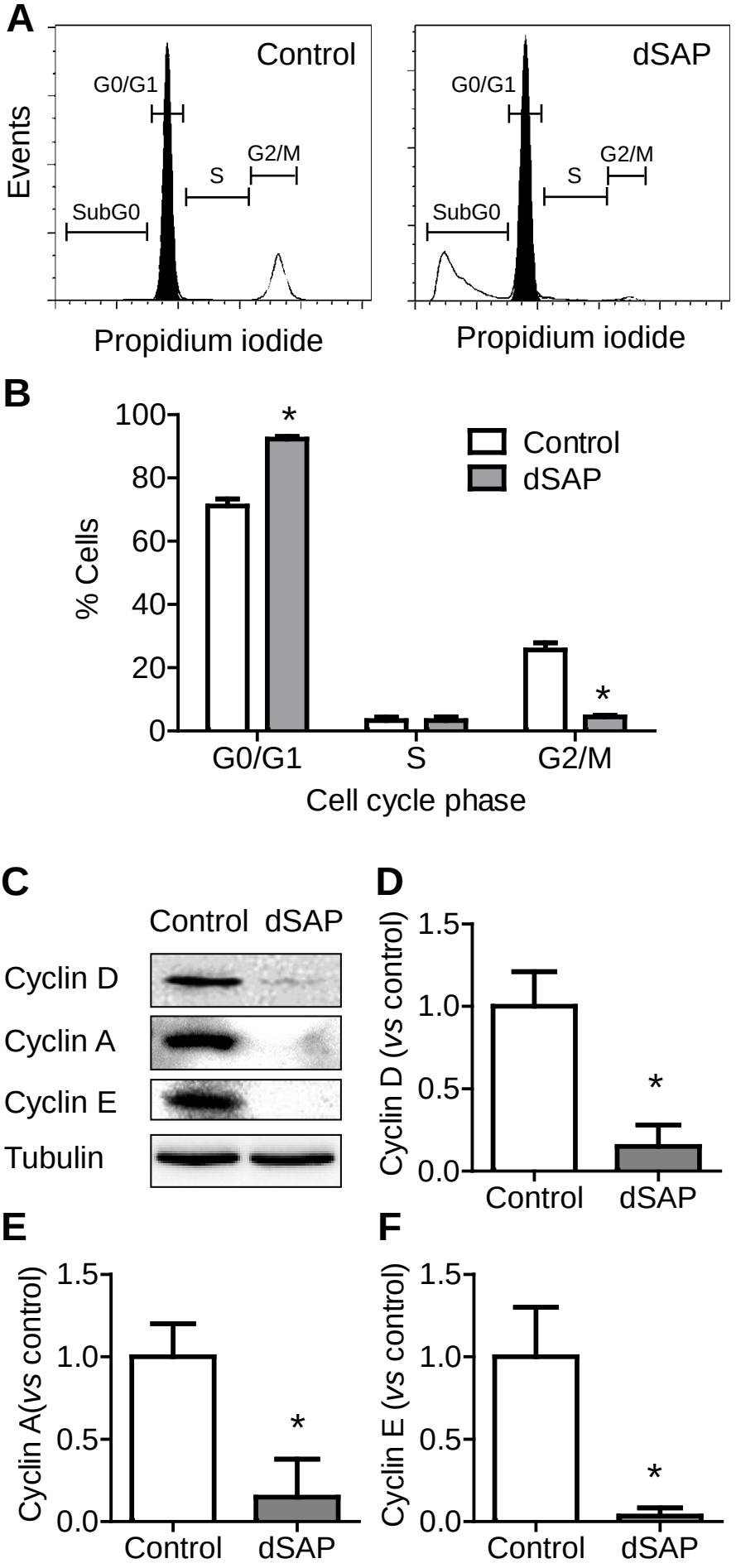


Fig. 6


A
Control
dSAP
G0/G1
S
G2/M
SubG0
Events
Propidium iodide
B
100
80
60
40
20
0
% Cells
Control
dSAP
*
G0/G1
S
G2/M
Cell cycle phase
C
Control
dSAP
Cyclin D
Cyclin A
Cyclin E
Tubulin
D
Cyclin D (vs control)
1.5
1.0
0.5
0.0
E
Cyclin A(vs control)
F
Cyclin E (vs control)

Supplementary Material

Saponins from edible spears of wild asparagus inhibit AKT, p70S6K, and ERK signalling, and induce apoptosis through G0/G1 cell cycle arrest in human colon cancer cells

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Table S1. Solutions of the *in vitro* digestion assay.

	Saliva-like solution	Gastric-like solution	Small intestinal-like solution (duodenum + bile)
Chemical reagents	2 mL NaCl 1.6 g/L	2 mL NaCl 8.17 g/L	12 mL $NaHCO_3$
	2 mL KCl 0.2 g/L	2 mL KCl 0.37 g/L	0.5 mL HCl 3.6 g/L
	2 mlLNaH_2PO_4 0.3 g/L		
	2 mL KH_2PO_4 0.05 g/L		
	0.2 mL $CaCl_2$ 0.14 g/L		
	0.2 mL $MgSO_4$ 1.23 g/L		
	0.2 mL $NaHCO_3$ 2.01 g/L		
Enzymes	0.1 mL α-amylase 1 mg/mL	0.2 mL pepsin 40 g/L	0.5 mL pancreatin 4 g/L
			0.5 mL bile 25 g/L
pH	6.8	2.0	8.0

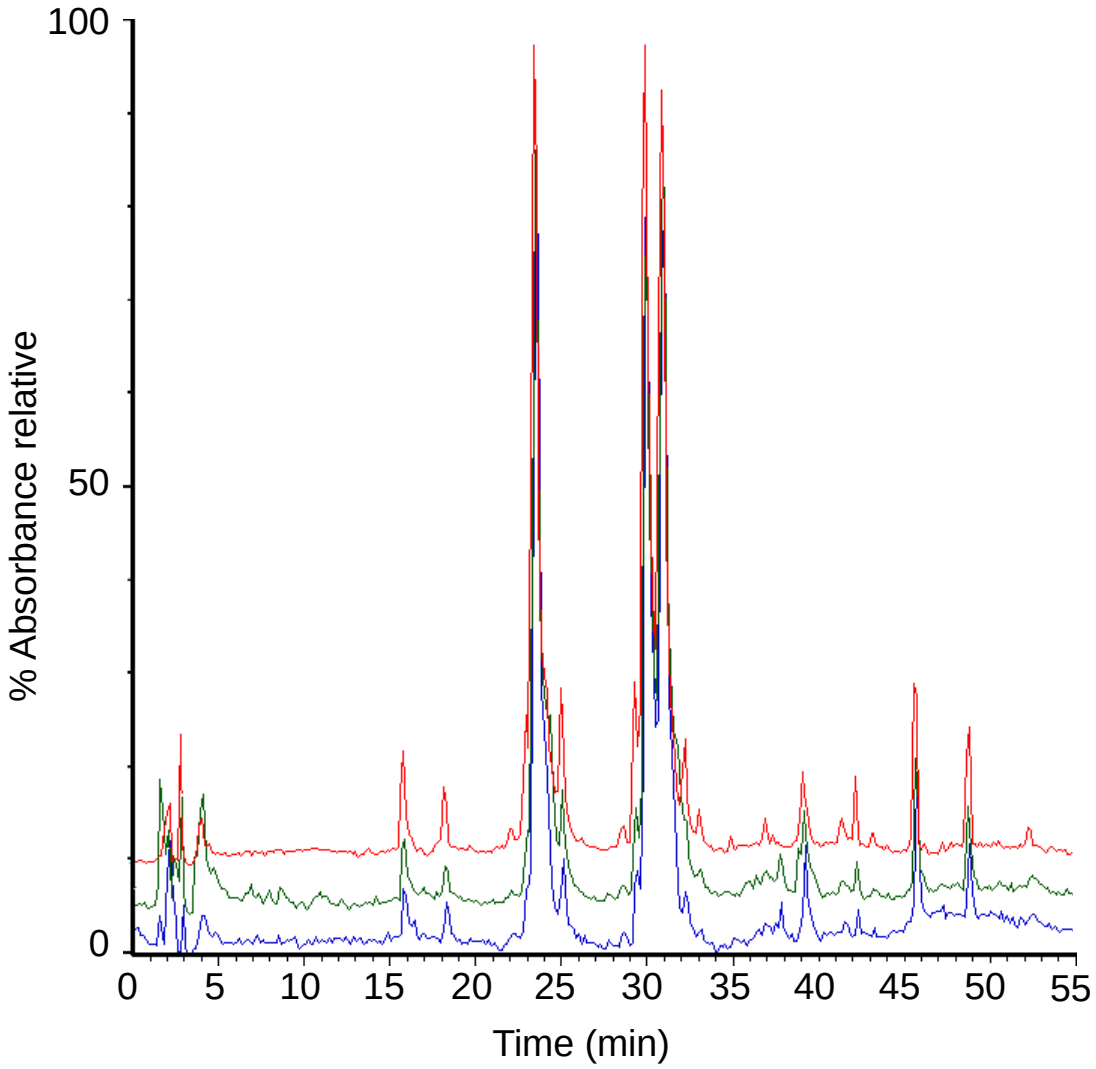

Fig. S1. Chromatograms of steroidal saponins in fractions from edible pears of *triguero* HT asparagus following different phases of simulated digestion. Relative absorbance of the native (red line), gastric digested (green line), and intestine digested (blue line) extracts showing the main peaks corresponding to the steroidal saponins. All lines started at the same level of relative absorbance, however, for the sake of clarity the distinct lines have been moved along the Y-axis.