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FULL PAPER

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In Vitro Toxicity of Asparagus Saponins in Distinct Multidrug-Resistant Colon Cancer Cells

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Colorectal cancer is the third most common cancer in the world. Many efforts have focused on finding natural molecules with potential chemo-preventive activity due to their low toxicity compared to synthetic drugs. However, comprehensive information on the bioactive fractions and components is still missing. In this study, we developed a method for the quantitative separation and isolation of saponins from asparagus genotypes consisting of an adsorption chromatography and subsequent liquid chromatographic separation on a reversed-phase column. The saponins isolated were tested for their cytotoxic activity against human colon cancer cell lines, which could develop cross-resistance to a wide variety of chemotherapeutic drugs. Our results showed that Huétor-Tájar asparagus saponins (HTSAP), mainly protodioscin and HTSAP-10 have higher cytotoxic activity than HTSAP-1, HTSAP-6, and HTSAP-8. This study links the potential anticancer effect of asparagus to specific saponins and unveils the triguero Huétor-Tájar asparagus as a nutraceutical particularly in colon cancer therapies.

Keywords: asparagus, saponins, colon cancer cells, cytotoxicity, biological activity.

Introduction

Increased fruit and vegetable intake is associated with reduced incidence of cardiovascular disease, cancer, and neurodegenerative disorders, among other chronic conditions.[1,2] These health-promoting effects are attributed to the wide broad of phytochemicals produced by plants, which includes polyphenolic compounds, saponins, oligo- and polysaccharides, and thiols among others.[3,4]

Cancer is one of the main causes of death worldwide, being colorectal malignancy the third most common type of cancer in modern countries.[5] It is a highly treatable and often curable disease when localized to the bowel. Surgery is the primary form of treatment and results in cure in approximately 50% of the patients. However, in the other 50% chemotherapy fails due to the apparition of drug-resistant tumor cells which are often the ultimate cause of death.[6]

MDR is one of the main problems to the successful treatment of colon cancer and consists of simultaneous resistance to many structurally and functionally unrelated cytotoxic agents. MDR in tumors involves different processes which have been broadly classified into non-cellular and cellular resistance mechanisms.[7] Nowadays, great efforts are being made in order to find new drugs against MDR colorectal tumors. One of the new trends in novel therapies against cancer consists in the development of steroid prodrugs, such as glucocorticoids.[8] This new strategy can improve the efficacy of the parent drugs and reduce the systemic or unwanted tissue/organ toxicity. These compounds are steroid hormones that are either secreted from the adrenal gland during exposure to acute and chronic stressors or administered pharmacologically to reduce inflammation and currently they are been used as new therapy mainly from solid

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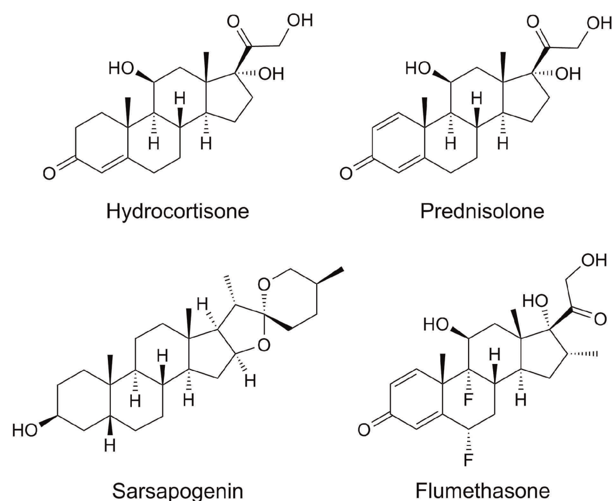

Figure 1. Chemical structures of sarsapogenin and the glucocorticoids, hydrocortisone, prednisolone, and flumethasone.

tumors.[9] Plant steroids share structural similarities with glucocorticoids (*Figure 1*).

They have a fundamental structure of four carbon rings called the steroid nucleus. The additions of chemical groups at distinct positions of the backbone are responsible of the multiple natural saponins such as solasodine, diosgenin, boswellic acid, glycyrrihizinic acid, guggulsterone, or sarsasapogenin.[10] From a nutraceutical point of view, several edible vegetables have been of interest due to their content in steroidal saponins.[11] Asparagus is one of the few sources of these compounds in the human diet.[12,13] In *Asparagus officinalis* L., the only cultivated *Asparagus* species for consumption with high economic importance nowadays, PD (26-*O*-β-D-glycopyranosyl-22-hydroxyfurost-5-ene-3β,26-diol-3-*O*-β-diglucorhamnoside) is the main saponin described in the spears. It is a furostanol steroidal saponin, with a pentacyclic sapogenin linked to two glucosyl and two rhamnosyl units.[14] However, a commercial Spanish asparagus, landrace HT asparagus which is a subspecies from hybridization between *A. officinalis* and *A. maritimus*,[15] has a saponin profile significant different of that from *A. officinalis*.[16] Several in vitro studies have demonstrated cytotoxic and antitumor properties of saponins from *Asparagus* genus on various tumor cells. The saponins extracted from the edible part of green *A. officinalis* inhibited the growth of HL-60 human leukemia cells in dose and time dependent manner,[17] while the saponin extract of white asparagus activates TNF-related apoptosis-inducing ligand (TRAIL) apoptotic pathway in human colon carcinoma cells and inhibits carcinogenesis in rat preclinical model.[18] Recently, it has been reported that a mixture of steroidal saponins isolated from HT asparagus exert anticancer effects via modulation of cell cycle in G_0/G_1 phase and triggering apoptosis in colon cancer HCT-116 cells.[19] In addition to their organoleptic, nutritional, and functional value, these asparagus from Spain may represent a new source of saponins with functional interest due to their cytotoxic activity. In order to unveil the importance of developing autochthonous asparagus genotypes with improved functional activity, we aimed to elucidate the effect of single saponins purified from HT asparagus genotypes on distinct chemo-resistant colon cancer cells. Consistent with this purpose we have developed a method to isolate the most important saponins present in HT asparagus.

Results and Discussion

Isolation of Saponins from HT Asparagus Extract

Asparagus is a commonly consumed vegetable known to contain significant concentrations of health-beneficial phytochemicals, notably a flavonoid, rutin, and the saponin PD.[16,20] In previous research, we showed that the phytochemical profile depends on the *Asparagus* species, then we hypothesized that the health-promoting potential of crop could be different. The edible part of the HT asparagus genotypes was found to provide high amounts of furostanol steroidal saponins that differ from protodioscin[16] and with a similar saponin profile to wild *Asparagus* species.[21] Firstly, and according to the method developed previously,[22] we prepared the crude saponins from the selected genotypes by extracting plant material with 80 % EtOH and using a non-ionic resin Amberlite XAD-16 column which was eluted with consecutive solvents of decreasing polarity to obtain the crude saponin fraction (40 % EtOH). Briefly, 1 kg of each HT genotype selected was extracted with 3 L of 80 % EtOH. Then, the concentrated extracts (500 mL) were passed through an Amberlite XAD-16 column (bed volume, 170 mL). Column was eluted with increasing percentages of ethanol in water: water (1 L), 20 % EtOH (1 L), 40 % EtOH (5 L), and 96 % EtOH (1 L). The resulting eluent (saponins fraction) with 40 % EtOH was collected and evaporated to yield dried powders. The saponin recovery was 4.5 g of saponins per kg fresh asparagus. This value of saponins extraction is higher than that described by other authors who studied for the complete extraction of saponins from various plant materials.[23,24] Hence, here we also tried to develop

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Table 1. Saponins identified by HPLC/MS (+/−) of triguero HT asparagus. Each compound was isolated and the ion fragmentation pattern was analyzed three times ($n=3$).

Saponin	t_R[a] [min]	MW[b]	Molecular ion [m/z] $[M-H]^-$	$[M+Na]^+$	Ion fragmentation Negative mode	Positive mode
HTSAP-1	21	1052	1051	1075	−Pen919−Hex757−Hex595−Hex433[c]	$[M-H_2O+H]$1035−Hex873−Hex711−Pen579−Hex417
HTSAP-3	25	1022	1021	1045	−Pen889−Pen757−Hex595−Hex433[c]	$[M-H_2O+H]$1005−Pen873−Hex711−Pen579−Hex417
PD	27	1048	1047	1071	−DoHex901−DoHex755−Hex593−Hex431[c]	$[M-H_2O+H]$1031−Hex869−DoHex723−DoHex577−Hex415
HTSAP-6	28	1036	1035	1059	−Pen903−DoHex757−Hex595−Hex433[c]	$[M-H_2O+H]$1019−DoHex873−Pen741−Hex579−Hex417
HTSAP-8	30	904	903	927	−DoHex757−Hex595−Hex433[c]	$[M-H_2O+H]$887−Hex725−DoHex579−Hex417
HTSAP-10	27	1048	1047	1071	−DoHex901−DoHex755−Unk595−Hex433[c]	$[M-H_2O+H]$1031−Hex869−DoHex723−DoHex577−Unk417

[a] t_R: Retention time. [b] MW: molecular weight. [c] Genin; Pen: Pentose; DoHex: Deoxyhexose; Hex: Hexose; Unk: Unknown.

the purification method to obtain high levels of saponin yields from asparagus.

Next, the crude saponins mixture was re-dissolved in 80% EtOH for further purification. A preliminary saponin fingerprint of the crude extract of HT genotypes was obtained by HPLC/MS analysis.[22] We have developed a HPLC-based method for the isolation of individual saponins based on the polar nature of their glyosidic chains. According to the analytical method,[22] the separation process was scaled up to semi-preparative reversed-phase chromatography to obtain a significant quantity of individual saponins. We optimized the flow rate and the mobile phase composed of acetonitrile/water (80:20). HPLC/MS experiments using the most intense peaks in the HPLC/MS spectrum were carried out to purify the individual saponins. Six different saponins were isolated based on the retention time and the genotypes sources used for their separation. All the structures were confirmed using the method developed by Vázquez-Castilla.[22] The fragmentation pattern is shown in *Table 1* and we found that all the saponins tested are furostanol genins with different sugar chains. In particular, the saponins isolated showed in the HPLC/MS spectrum a principal precursor $[M-H]^-$ ion, whose fragmentation pattern allowed to identify the occurrence of sugar units (pentose, deoxyhexose, and hexose) linked to the genin by checking neutral losses of 132, 146, and 162 a.m.u., corresponding to different combinations of pentose, deoxyhexose, and hexose units, respectively. The positive mode spectra were coincident with the fragmentation described (*Table 1*). The HT-33 genotype was used as the source of HTSAP-1, HTSAP-8, and HTSAP-10; HT-2 genotype was selected for isolating HTSAP-3; HT-17 genotype was used for isolating HTSAP-6; and HT-53 genotype was used as the source of PD. This amended protocol would be applicable to separate all saponins detected in *Asparagus* species with a feasible recovery.

Cytotoxic Activity

The cytotoxic activity of the six saponins isolated from HT asparagus was tested for three distinct colon cancer cell lines (HCT-116, HT-29, and Caco-2) by using the MTT assay, a method for screening anti-proliferative agents. *Figure 2* shows cell viability dose-response curves for each saponin after cells were incubated for 48 h. 5-Fluorouracil (5-FU), the most commonly used drug in colon cancer therapy, was used as positive control.[25] HTSAP-10 and PD were more cytotoxic than the other saponins tested in the three cell lines. Remarkably, HTSAP-10 was cytotoxic against HCT-116, HT-29, and Caco-2 cell lines with the IC_{50} values of 6.6±0.2, 101.2±3.8, and 76.3±6.1 μM, respectively. Since IC_{50} for HTSAP-10 in HCT-116 was below 10 μM, this saponin can be considered as active in HTC-116 cells. The cytotoxic effect of the HTSAP-10 was higher than the effect of 5-FU only in HTC-116 cells (75±5 μM for HCT-116 cells, 62±1 μM for HT-29 cells, and 14±0.8 μM for Caco-2 cells). PD was cytotoxic against HCT-116 and HT-29 cell lines, and the IC_{50} values were 19.0±0.9 and 13.3±0.4 μM, respectively. It is of interest that these IC_{50} values were lower than the IC_{50} values for 5-FU in the same cell lines. HTSAP-1 only reduced the viability of the HTC-116 cell

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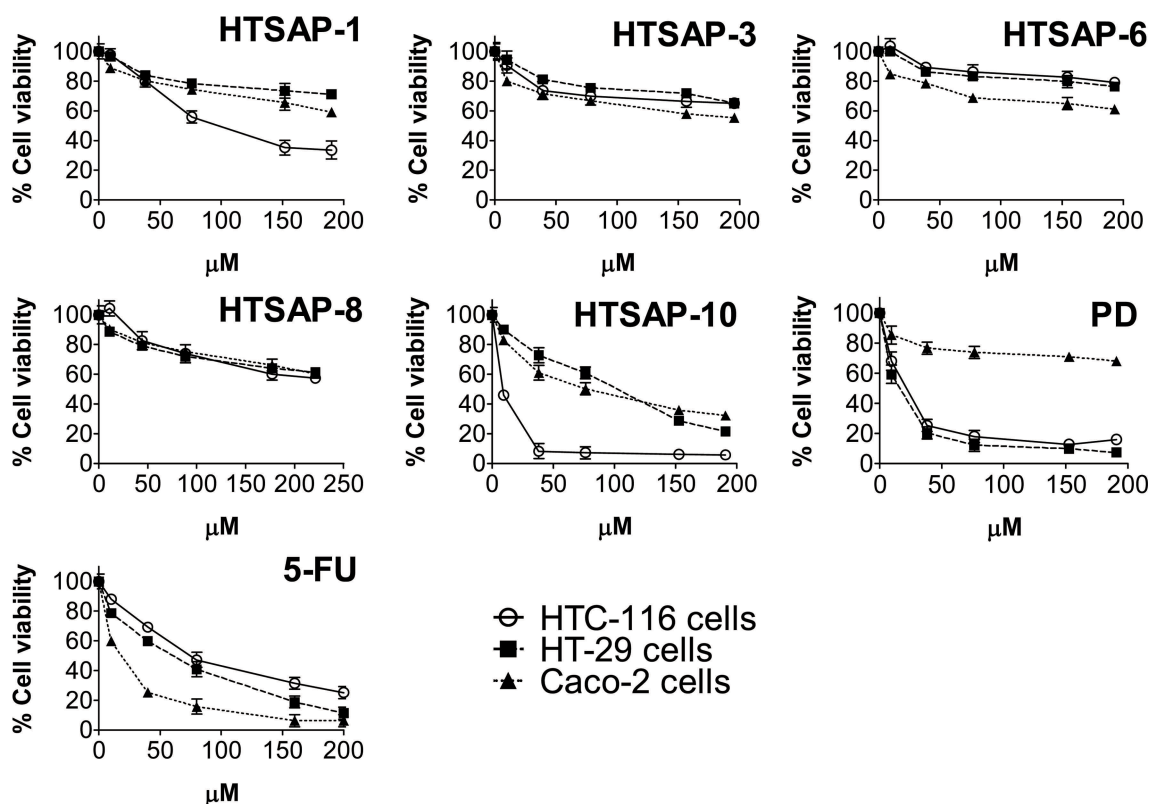

Figure 2. Cytotoxic effect of individually saponins isolated from HT asparagus on different colon cancer cells line. Cells were treated with increasing concentrations (0–250 µм) of HSAP-1, HSAP-3, HSAP-6, HSAP-8, HSAP10, PD, and 5-FU for 48 h. All assays were carried out in three separate trials, each experiment included five replicate sets. Data were normalized to control cells (set at 100 %) and were shown as mean ± SD.

line with an IC_{50} of 89.3 ± 2.8 µм. In contrast, HTSAP-3, HTSAP-6, and HTSAP-8 did not affect the viability of any of the cell lines tested. In comparison to the IC_{50} obtained for the total extract of HT asparagus (76 µg/mL) in HCT-116 cells,[19] our results show that PD and HTSAP-10 have higher cytotoxic activity (21 ± 1.1 and 7 ± 0.2 µg/mL which correspond to 19.0 ± 0.9 and 6.6 ± 0.2 µм, respectively). These results could suggest that the other saponins may be diluting the cytotoxic effect of the HTSAP-10 in the total extract. However, a possible antagonistic effect of different saponins should not be excluded. It is interesting to point out that, small modifications in the structure of the saponins tested influence significantly the cytotoxic activity.[26,27] In fact, all the saponins isolated but PD shared the same smallest fragmentation ion 433. The product ion at *m/z* 431 from PD was due to the cleavage of additional inner hexose moiety and corresponding to the deprotonated protodiosgenin (*Figure 3*).[22] Therefore, the 433 fragment is compatible with the presence of a furostanol genin with a single bond between carbons 5 and 6 in ring B (*Table 1*) and only HTSAP-10 showed significant activity.

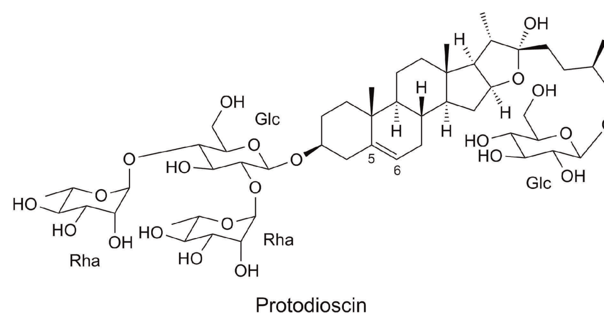

Figure 3. Chemical structure of protodioscin.

Because HTSAP-10 and PD were the most cytotoxic saponins, our results suggest that the sugar side chain was the key factor for the cytotoxic activity, specially the kind of sugar. According to this hypothesis, the presence of two deoxy sugars in HTSAP-10 and PD provokes an acute decrease in the viability of HCT-116 cells. In fact, Bell[28] had reported that certain MDR cell

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lines were particularly sensitive to 2-deoxy-D-glucose toxicity. Since MDR is the major cause for the reduced effects of chemotherapy, MDR cells lines were used for the identification of novel antitumor compounds.[29] In order to find the best strategy for selecting the suitable agents for chemo-resistant tumors, in this research we used three different colon cancer cell lines and first we established the resistance of these cells using increasing concentrations of the anticancer drug, 5-FU. The results showed that Caco-2 cells were less resistant to this anticancer drug, while the other two lines could be used as models for the MDR colon cell lines. When we exposed the three lines to the different saponins, we found that, remarkably, HTSAP-10 showed to be more effective against the HCT-116 cells, with important application in MDR lines. Also, PD showed higher efficiency in HCT-116 and HT-29 cells than 5-FU. The mutation status of cancer critical genes is different in HCT-116, HT-29, and Caco-2 cells.[30] These mutations could be associated with the higher resistance of HCT-116 and HT-29 cells to 5-FU compared to Caco-2 cells and the behavior to the HTSAP-10 and PD. The number of studies regarding the cytotoxic effects of PD is large. It inhibits DNA synthesis in human leukemia cell line HL-60,[17] and even at low concentrations (lower than 2 μM), displays antitumor activity against colon cancer and melanoma cell lines HCT-116, SW-620, and LOX IMV, amongst others.[31] Due to its cytotoxic activity, PD has been proposed to develop a novel mechanism of anticancer action.[32] The source of PD has been *Tribulus*,[33] *Trigonella* (fenugreek),[34] and *Dioscorea* genus.[35] To the best of our knowledge, we demonstrated for the first time that PD and other furostanol saponin, HTSAP-10, with high cytotoxic activity against colon cancer cells, were obtained from HT asparagus. Of note, we have previously published that these saponins are resistant to simulated digestion[19] and due to their poor intestinal absorption,[36] they are colon-accessible, where they may exert their biological effects. This represent an advantage respect to 5-FU that suffers a reduction of cellular drug uptake because water-soluble drugs may attach to transporters carrying nutrients and therefore fail to accumulate in the colonic tumoral cells at relevant concentrations.[7,37,38]

We have previously demonstrated that the total extract of HT asparagus interfered with MEK, mTOR, and PI3 K pathways by inhibiting the phosphorylation of ERK, p70S6K, and AKT proteins HCT-116 cells which presented a dietary strategy against colon cancer cells.[19] Indeed, dual targeted inhibition of MEK and PI3 K pathway effectors presents a potential strategy to overcome resistance to MEK inhibitor therapy.[39] HCT-116, Caco-2, and HT-29 cells have a different mutation profile. In HCT-116 cells, KRAS, and PIK3CA are constitutively activated.[30] HT-29 cells also contain a PIK3CA mutation but unlike to HCT-116 cells, HT-29 cells have a mutation on BRAF and a mutation in TP53. In contrast, Caco-2 cells are characterized to be wild type on BRAF, KRAS, and PIK3CA but TP53 contains a deletion and an early termination signal. This mutation has been shown to play an important role on MDR genes.[40,41] Despite the number of mutated genes could be related to the resistance of these cells to saponins and other chemotherapeutic drugs, it is of great interest to deepen in the mechanisms by which HT saponins may interfere with the constitutively activated pathways in these cell lines.

Conclusions

In this research, we optimized an effective and easy method for the extraction and isolation of individual saponins from different asparagus genotypes by means of extraction with aqueous ethanol, purification by adsorption chromatography with Amberlite XAD-16 resin, separation by reversed-phase chromatography, electrospray ionization, and detection in a single quadrupole mass analyzer. Furthermore, we made a screening of the main saponins detected in HT asparagus as a new alternative phytochemical agent against colon cancer cells. We found that HTSAP-10 and PD displayed potent cytotoxic effects, even greater than that of 5-FU, on the cell lines selected, opening new perspectives for the combined use of both saponins in MDR lines. Both saponins share similar structure and can be easily obtained from the main HT asparagus genotypes. Also, these results give us clues to modify the structure of synthetic drugs for the treatment of cancer. Although further research on the mechanisms of action of the furostanol saponins on molecular and cellular levels are needed, this study provided valuable information for future breeding programs since HT genotypes could be considered as a new source of possible agents for the treatment of chemo-resistant tumors.

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Experimental Section

Abbreviations

5-FU: 5-Fluorouracil; AKT (or PKB): protein kinase B; BRAF: B-Raf serine/threonine kinase; ERK: extracellular signal-regulated kinase; HT: triguero Huétor-Tájar asparagus landrace; HTSAP: Huétor-Tájar saponin; MDR: multidrug resistance; KRAS: rat sarcoma viral oncogene homologue; mTOR: mammalian target of rapamycin; PD: protodioscin; PI3 K: phosphatidylinositol-3-kinase pathway; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; TRAIL: TNF-related apoptosis-inducing ligand; TP53: tumor protein TP53.

Chemicals and Reagents

PD (97% purity checked by NMR) was purchased from Chromadex Chemical Co. (Barcelona, Spain). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide), 5-FU, and Amberlite XAD-16 polymeric resin were purchased from Sigma–Aldrich (Madrid, Spain). EtOH, formic acid (96%), and acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pure deionized water was obtained from a Milli-Q 50 system (Millipore Corporation, Bedford, MA, USA). All cell culture reagents were purchased from Gibco (Madrid, Spain).

Plant Material

The HT asparagus is also known as 'Morado de Huetor' and the only tetraploid asparagus cultivar, together with the Italian 'Violetto d'Albenga' in Europe.[42] Previous studies on strain identification phylogeny had suggested that the triguero HT asparagus was close to *Asparagus officinalis* and *A. maritimus*.[15] Since 2000, the European Commission registered the triguero HT asparagus as protected geographical indication in the European Union.[43] Only fresh spears of the triguero asparagus, harvested from HT landrace, Granada (Spain), were used in the present study. The samples evaluated consisted on spears from HT asparagus selected by the application of a clustering analysis in a previous research based on the total quantity and characteristic saponin profile.[16,22] The selected genotypes let to isolate some individual saponins. All the spears were harvested from experimental fields in controlled conditions (Granada, Spain). Samples were immediately transported to the laboratory where they were trimmed to a final length of 21 cm, weighed, and frozen at −20 °C.

Preparation of Saponins Extract

Samples of fresh asparagus were extracted with 80% EtOH in a ratio of 3 l/kg in a bench top electrical food processor (Thermomix TM 21; Vorwerk, Madrid, Spain) for 8 min at maximum speed and filtered. The supernatants were concentrated to dryness, dissolved in water, and loaded in a column packed with Amberlite XAD16. The column volume was chosen considering a ratio of resin 170 mL/kg of fresh asparagus. Elution was performed with gradient solutions (water, 20% EtOH, 40% EtOH, 96% EtOH) using a ratio 1:4 (v/v) of filler. The saponins fraction eluted with 40% EtOH. All extractions were made in triplicate. The dried ethanol extract was re-dissolved in 50 mL of 80% EtOH and 1 mL of this dissolution was centrifuged at 2500*g* for 3 min and injected into the HPLC/MS system.

Saponin Analysis by HPLC/MS

The method for saponin was described previously in detail.[22] Briefly, an HPLC Waters Alliance system fitted to a Mediterranean Sea18 reversed-phase analytical column (25 cm length ×4.6 mm i.d., 5 μm particle size; Teknokroma, Barcelona) was used. An elution gradient was used with solvent A (water with 1% formic acid) and B (acetonitrile with 1% formic acid): 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 saponins were detected using an online connected quadrupole mass analyzer (ZMD4, Micromass, Waters, Inc., Manchester, U.K.). Electrospray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 V (negative mode) and 50 V (positive mode) with scans from *m/z* 200 to 1200. Capillary voltage was 3 kV; dessolvation temperature was 200 °C; source temperature 100 °C and extractor voltage 12 V. The flow rate was kept at 1 mL/min and a split ratio was 5:1 for each analysis.

Isolation of Saponins

A semi-preparative Synergi 4 μm Hydro-RP 80A reversed-phase column (25 cm length×21.20 mm i.d., 4 μm particle size; Phenomenex, Macclesfield, Cheshire, U.K.) was used for the isolation of individual saponins. The flow rate was maintained at 20 mL/min, and the injection volume was 400 μL. Before the MS detector, the flow was split with a T-connection (0.4 mL/min to the MS detector and 19.4 mL/min to the collection port). The saponin-rich fractions were evaporated, dissolved in 1 mL of 80% EtOH, centri-

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fuged at 2500*g* for 3 min and re-injected into an analytical column of HPLC/MS system.

Quantitative Analysis

The external standard method was used for the quantification of asparagus saponins.[22] For each standard, PD and shatavarin, the selected ion chromatogram corresponding to its molecular ion in negative mode at 100 V was integrated and the peak area was plotted against concentration and subjected to regression analysis.

Colon Cancer Cell Lines

Three colon cancer cell lines with different degree of sensitivity to 5-FU were included in the present study.[25] HCT-116, HT-29, and Caco-2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture media were as follows: HCT-116 and HT-29: McCoy's 5 A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 172 μM streptomycin; Caco-2: Dulbecco's modified Eagle essential medium (DMEM), 10% FBS, 1% non-essential amino acids, 100 U/mL penicillin, and 172 μM streptomycin. All cell lines were cultured at 37 °C and 5% CO_2 incubators.

Cell Viability Assay

Cell viability was assayed based on the ability of live cells to reduce MTT. HCT-116, HT-29, and Caco-2 human colon cancer 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 different concentrations of individual saponins (ranging from 0 to 250 μM) in DMSO (<0.1%). PD isolated from green asparagus commercial hybrids was used to compare the effect on cell viability with HT saponins.[16]

Cells were incubated for 48 h, and then 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well. Cells were incubated for 3 h at 37 °C in a humidified chamber with 5% CO_2. The supernatant was then sucked off, after which 100 μL of DMSO were added to each well. The culture plate was gently agitated (about 30 min) to make purple formazan dissolve completely. The concentration of formazan was measured spectrophotometrically at 490 nm using a Multiskan Spectrum microplate reader (ThermoLabsystems, MA, USA). All MTT assays were carried out in three separate trials, and each experiment included five replicate sets.

Statistical Analysis

The experiments were replicated at least three times with five replicates wells in each concentration. The results were computerized and analyzed using Statistical Package for Social Science (SPSS Inc., Version 17, Chicago, IL, USA). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's post-hoc test. The P value <0.05 was deemed to be statistically significant. Cell viability data was normalized to control cells (set at 100%). The normalized data was then plotted using the GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA) and the IC_{50} (median inhibitory concentration) for each saponin and each cell line was calculated from this.

Author's Contribution Statement

Sara Jaramillo-Carmona collected test data, interpreted the results, and drafted the manuscript. Rocío Rodríguez-Arcos and Ana Jiménez-Araujo helped to draft the manuscript. Sara Jaramillo-Carmona, Rafael Guillén-Bejarano, and Sergio López designed the study, interpreted the results, and performed the statistical analyses.

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References

[1] J. Chantal, M. Touvier, C. Lassale, L. Fezeu, P. Galan, S. Hercberg, E. Kesse-Guyot, 'Cluster analysis of polyphenol intake in a French middle-aged population (aged 35–64 years)', *J. Nutr. Sci.* **2016**, *5*, e28.
[2] P. Manna, S. K. Jain, 'Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies', *Metab. Syndr. Relat. Disord.* **2015**, *13*, 423–444.

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[3] S. K. Chang, C. Alasalvar, F. Shahidi, 'Review of dried fruits: Phytochemicals, antioxidant efficacies, and health benefits,' *J. Funct. Foods* **2016**, *21*, 113–132.

[4] C. J. Dillard, J. B. German, 'Phytochemicals: nutraceuticals and human health', *J. Sci. Food Agric.* **2000**, *12*, 1744–1756.

[5] J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman, F. Bray, http://www.wcrf.org/int/cancer-facts-figures/worldwide-data, GLOBOCAN 2012 v1.1, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet], Lyon, France, International Agency for Research on Cancer, 2014. Available from: http://globocan.iarc.fr, accessed on 16/01/2015.

[6] A. K. Mitra, V. Agrahari, A. Mandal, K. Cholkar, C. Natarajan, S. Shah, M. Joseph, H. M. Trinh, R. Vaishya, X. Yang, Y. Hao, V. Khurana, D. Pal, 'Novel delivery approaches for cancer therapeutics', *J. Controlled Release* **2015**, *219*, 248–268.

[7] T. Hu, Z. Li, C.-Y. Gao, C. H. Cho, 'Mechanisms of drug resistance in colon cancer and its therapeutic strategies', *World J. Gastroenterol.* **2016**, *22*, 6876–6889.

[8] M. O. F. Khan, K. K. Park, H. J. Lee, 'Antedrugs: An approach to safer drugs', *Curr. Med. Chem.* **2005**, *12*, 2227–2239.

[9] M. A. Pufall, 'Glucocorticoids and Cancer', *Adv. Exp. Med. Biol.* **2015**, *872*, 315–333.

[10] S. S. Patel, J. K. Savjani, 'Systematic review of plant steroids as potential anti-inflammatory agents: Current status and future perspectives', *J. Clin. Pharmacol.* **2015**, *4*, 121–125.

[11] P. Kuppusamy, M. M. Yusoff, G. P. Maniam, S. J. A. Ichwan, I. Soundharran, N. Govindan, 'Nutraceuticals as potential therapeutic agents for colon cancer: a review', *Acta Pharm. Sin. B* **2014**, *4*, 173–181.

[12] U. Sharma, R. Saini, Bobita, R. N. Kuma, B. Singh, 'Steroidal saponins from *Asparagus racemosus*', *Chem. Pharm. Bull.* **2009**, *57*, 890–893.

[13] E. J. Lee, K. S. Yoo, B. Patil, 'Development of a rapid HPLC-UV method for simultaneous quantification of protodioscin and rutin in white and green asparagus spears', *J. Food Sci.* **2010**, *75*, 703–709.

[14] A. Schwarzbach, M. Schreiner, D. Knorr, 'Effect of cultivars and deep freeze storage on saponin content of white asparagus spears (*Asparagus officinalis* L.)', *Eur. Food Res. Technol.* **2006**, *222*, 32–35.

[15] R. Moreno, J. A. Espejo, A. Cabrera, J. Gil, 'Origin of tetraploid cultivated asparagus landraces inferred from nuclear ribosomal DNA internal transcribed spacers' polymorphisms', *Ann. Appl. Biol.* **2008**, *15*, 233–241.

[16] S. Vázquez-Castilla, S. Jaramillo-Carmona, J. M. Fuentes-Alventosa, A. Jiménez-Araujo, R. Rodríguez-Arcos, P. Cermeño-Sacristán, J. A. Espejo-Calvo, R. Guillén-Bejarano, 'Saponin profile of green asparagus genotypes', *J. Agric. Food Chem.* **2013**, *61*, 11096–11108.

[17] Y. Shao, C.-K. Chin, C.-T. Ho, W. Ma, S. A. Garrison, M.-T. Huang, 'Anti-tumor activity of the crude saponins obtained from asparagus', *Cancer Lett.* **1996**, *104*, 31–36.

[18] S. Bousserouel, J. Le Grandois, F. Gossé, D. Werner, S. W. Barth, E. Marchioni, J. Marescaux, F. Raul, 'Methanolic extract of white asparagus shoots activates TRAIL apoptotic death pathway in human cancer cells and inhibits colon carcinogenesis in a preclinical model', *Int. J. Oncol.* **2013**, *43*, 394–404.

[19] S. Jaramillo, F. J. G Muriana, R. Guillén, A. Jiménez-Araujo, R. Rodríguez-Arcos, S. López, 'Saponins from edible spears of wild asparagus inhibit AKT, p70 S6 K, and ERK signalling, and induce apoptosis through G0/G1 cell cycle arrest in human colon cancer HCT-116 cells', *J. Funct. Foods* **2016**, *26*, 1–10.

[20] J. M. Fuentes-Alventosa, S. Jaramillo, G. Rodríguez-Gutierrez, P. Cermeno, J. A Espejo, A. Jiménez-Araujo, R. Guillén-Bejarano, J. Fernández-Bolanos, R. Rodríguez-Arcos, 'Flavonoid profile of green asparagus genotypes', *J. Agric. Food Chem.* **2008**, *56*, 6977–6984.

[21] S. Jaramillo-Carmona, R. Rodriguez-Arcos, A. Jiménez-Araujo, S. López, J. Gil, R. Moreno, R. Guillén-Bejarano, 'Saponin Profile of Wild Asparagus Species', *J. Food Sci.* **2017**, *82*, 638–646.

[22] S. Vázquez-Castilla, S. Jaramillo-Carmona, J. M. Fuentes-Alventosa, A. Jiménez-Araujo, R. Rodríguez-Arcos, P. Cermeño-Sacristán, J. A. Espejo-Calvo, R. Guillén-Bejarano, 'Optimization of a method for the profiling and quantification of saponins in different asparagus genotypes', *J. Agric. Food Chem.* **2013**, *61*, 6250–6258.

[23] B. Sarvin, E. Stekolshchikova, I. Rodin, A. Stavrianidi, O. Shpigun, 'Optimization and comparison of different techniques for complete extraction of saponins from *T. terrestris*', *J. Appl. Res. Med. Aromat. Plants* **2018**, *8*, 75–82.

[24] K. Kawano, K. Sakai, H. Sato, S. Sakamura, 'A Bitter Principle of Asparagus: Isolation and Structure of Furostanol Saponin in Asparagus Storage Root', *Agric. Biol. Chem.* **1975**, *39*, 1999–2002.

[25] K. Bracht, A. M. Nicholls, Y. Liu, W. F. Bodmer, '5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency', *Br. J. Cancer* **2010**, *103*, 340–346.

[26] S. Man, W. Gao, Y. Zhang, L. Huang, C. Liu, 'Chemical study and medical application of saponins as anticancer agents', *Fitoterapia* **2010**, *81*, 703–714.

[27] Y. Mimaki, A. Yokosuka, M. Kuroda, Y. Sashida, 'Cytotoxic activities and structure-cytotoxic relationships of steroidal saponins', *Biol. Pharm. Bull.* **2001**, *24*, 1286–1289.

[28] S. E. Bell, D. M. Quinn, G. L. Kellett, J. R. Warr, '2-Deoxy-D-glucose preferentially kills multidrug-resistant human KB carcinoma cell lines by apoptosis', *Br. J. Cancer* **1998**, *78*, 1464–1470.

[29] F. El Khoury, L. Corcos, S. Durand, B. Simon, C. Le Jossic-Corcos, 'Acquisition of anticancer drug resistance is partially associated with cancer sternness in human colon cancer cells', *Int. J. Oncol.* **2016**, *49*, 2558–2568.

[30] D. Ahmed, P. W. Eide, I. A. Eilertsen, S. A. Danielsen, M. Eknæs, M. Hektoen, G. E. Lind, R. A. Lothe, 'Epigenetic and genetic features of 24 colon cancer cell lines', *Oncogenesis* **2013**, *2*, 1–8.

[31] C. K. Chin, S. A. Garrison, C. T. Ho, Y. Shao, M. Wang, J. Simon, M. T. Huang, 'Functional elements from asparagus for human health', *Acta Hortic.* **2002**, *589*, 233–241.

[32] K. Hu, X. Yao, 'Protodioscin (NSC-698 796): Its spectrum of cytotoxicity against sixty human cancer cell lines in an anticancer drug screen panel', *Planta Med.* **2002**, *68*, 297–301.

[33] M. Ganzera, E. Bedir, I. A. Khan, 'Determination of steroidal saponins in *Tribulus terrestris* by reversed-phase high-

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performance liquid chromatography and evaporative light scattering detection’, *J. Pharm. Sci.* **2001**, *90*, 1752 – 1758.
[34] H. Hibasami, H. Moteki, K. Ishikawa, H. Katsuzaki, K. Imai, K. Yoshioka, Y. Ishii, T. Komiya, ‘Protodioscin isolated from fenugreek (*Trigonella foenumgraecum* L.) induces cell death and morphological change indicative of apoptosis in leukemic cell line H-60, but not in gastric cancer cell line KATO III’, *Int. J. Mol. Med.* **2003**, *11*, 23 – 26.
[35] M. Dong, X. Z. Feng, B. X. Wang, T. Ikejima, L. J. Wu, ‘Steroidal saponins from Dioscorea panthaica and their cytotoxic activity’, *Pharmazie* **2004**, *59*, 294 – 296.
[36] I. A. Cree, P. Charlton, ‘Molecular chess? Hallmarks of anticancer drug resistance’, *BMC Cancer* **2017**, *17*, 1 – 10.
[37] K. Yu, F. Chen, C. Li, ‘Absorption, Disposition, and Pharmacokinetics of Saponins from Chinese Medicinal Herbs: What Do We Know and What Do We Need to Know More?’, *Curr. Drug Metab.* **2012**, *13*, 577 – 598.
[38] Y.-K. Zhang, Y.-J. Wang, P. Gupta, Z.-S. Chen, ‘Multidrug Resistance Proteins (MRPs) and Cancer Therapy’, *AAPS J.* **2015**, *17*, 802 – 812.
[39] S. Temraz, D. Mukherji, A. Shamseddine, ‘Dual Inhibition of MEK and PI3 K Pathway in KRAS and BRAF Mutated Colorectal Cancers’, *Int. J. Mol. Sci.* **2015**, *16*, 22976 – 22988.
[40] M. E. Goldsmith, J. M. Gudas, E. Schneider, K. H. Cowan, ‘Wild type p53 stimulates expression from the human multidrug resistance promoter in a p53-negative cell line’, *J. Biol. Chem.* **1995**, *270*, 1894 – 1898.
[41] Y.-C. Lin-Lee, S. Tatebe, N. Savaraj, T. Ishikawa, M. T. Kuo, ‘Differential sensitivities of the MRP gene family and γ-glutamylcysteine synthetase to prooxidants in human colorectal carcinoma cell lines with different p53 status’, *Biochem. Pharmacol.* **2001**, *61*, 555 – 563.
[42] R. Moreno, J. A. Espejo, A. Cabrera, T. Millan, J. Gil, ‘Ploidic and molecular analysis of ‘Morado de Huétor’ asparagus (*Asparagus officinalis* L.) population: A Spanish tetraploid landrace’, *Genet. Resour. Crop Evol.* **2006**, *53*, 729 – 736.
[43] European Commission, ‘Commission Regulation (EC) No. 547/2000 of 14 March 2000 supplementing the Annex to Regulation (EC) No. 2400/96 on the entry of certain names in the “Register of protected designations of origin and protected geographical indications” provided for in Council Regulation (EEC) No. 2081/92 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs’, *Official J. Eur. Communities* **2000**, L67/43, 8 – 11.

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