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In vitro toxicity of asparagus saponins in distinct multidrug resistance colon cancer cells

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List of Abbreviations

5-FU: 5-Fluorouracil; AKT(or PKB): protein kinase B; BRAF: B-Raf serine/threonine kinase; ERK: extracellular signal-regulated kinase; HT: *triguero*

25 Huétor-Tájar asparagus landrace; HTSAP: Huétor-Tájar saponin; MDR:
26 multidrug resistance; KRAS: rat sarcoma viral oncogene homologue; mTOR:
27 mammalian target of rapamycin; PD: protodioscin; PI3K: phosphatidylinositol-3-
28 kinase pathway; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase,
29 catalytic subunit alpha; TRAIL: TNF-related apoptosis-inducing ligand; TP53:
30 Tumor protein TP53.

31

ABSTRACT

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 consist 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 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, glycyrrhizinic 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-3 β ,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 are a subspecies from hybridization between *A. officinalis* and *Asparagus 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 G₀/G₁

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 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, one 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

131 increasing percentages of ethanol in water: water (1 L), 20% EtOH (1 L), 40% 5
132 L), and 96% EtOH (1 L). The 40% EtOH resulting eluent (saponins fraction) was
133 collected and evaporated to yield dried powders. The saponin recovery was 4.5
134 g saponins per Kg fresh asparagus. This value of saponins extraction is higher
135 than described by others authors who studied for the complete extraction of
136 saponins from various plant materials [23-24]. Hence, here we also tried to
137 develop the purification method to obtain high levels of saponin yields from
138 asparagus.

139 Next, the crude saponins mixture was re-dissolved in 80% EtOH for
140 further purification. A preliminary saponin fingerprint of the crude extract of *HT*
141 genotypes was obtained by HPLC-MS analysis [22]. We have developed a
142 HPLC-based method for the isolation of individual saponins based on the polar
143 nature of their glycosydic chains. According to the analytical method [22], the
144 separation process was scaled up to semipreparative reverse phase
145 chromatography to obtain a significant quantity of individual saponins. We
146 optimized the flow rate and the mobile phase composed of acetonitrile/water
147 (80:20). HPLC-MS experiments using the most intense peaks in HPLC-MS
148 spectrum, were carried out to purify the individual saponins. Six different
149 saponins were isolated based on the retention time and the genotypes sources
150 used for their separation. All the structures were confirmed using the method
151 developed by Vázquez-Castilla [22]. The fragmentation pattern is shown in
152 Table 1 and we found that all the saponins tested are furostanol genins with
153 different sugar chains. In particular, the saponins isolated showed in the HPLC-
154 MS spectrum a principal precursor $[M-H]^-$ ion, whose fragmentation pattern
155 allowed to identify the occurrence of sugar units (pentose, deoxyhexose,
156 hexose) linked to the genin by checking neutral losses of 132, 146, 162 a.m.u.,
157 corresponding to different combinations of pentose, deoxyhexose and hexose

units, respectively. The positive mode spectrums 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 for 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 IC_{50} values of 6.6 ± 0.2 , 101.2 ± 3.8 and 76.3 ± 6.1 μ M, respectively. Since IC_{50} for *HTSAP-10* in HCT-116 was below 10 μ M, 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 μ M for HCT-116 cells, 62 ± 1 μ M for HT-29 cells, and 14 ± 0.8 μ M 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 μ M, 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 line with an IC_{50} of 89.3 ± 2.8 μ M. 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 μ g/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 μ g/mL which correspond to 19.0 ± 0.9 and 6.6 ± 0.2 μ M, 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 the B-ring (Table 1) and only *HTSAP-10* showed significant activity.

Due to *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 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 anti-tumoral 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 anti-cancer drug, *5-FU*. The results showed that Caco-2 cells were less resistant to this anti-cancer drug, while the others two lines could be used as models for the *MDR* colon cell lines. When we exposure the three lines to the different saponins we found that, remarkably, *HTSAP-10* showed to be more effective to 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].

218 These mutations could be associated with the higher resistance of HCT-116
219 and HT-29 cells to *5-FU* compared to Caco-2 cells and the behavior to the
220 *HTSAP-10* and *PD*. The number of studies regarding the cytotoxic effects of *PD*
221 is large. It inhibits DNA synthesis in human leukemia cell line HL-60 [17], and
222 even at low concentrations (lower than 2 μ M), displays anti-tumoral activity
223 against colon cancer and melanoma cell lines HCT-116, SW-620 and LOX IMV,
224 amongst others [31]. Due to its cytotoxic activity, *PD* has been proposed to
225 develop a novel mechanism of anticancer action [32]. The source of *PD* has
226 been *Tribulus* [33], *Trigonella* (fenugreek) [34] and *Dioscorea* genus [35]. To the
227 best of our knowledge we demonstrated for the first time that *PD* and other
228 furostanol saponin, *HTSAP-10*, with high cytotoxic activity against colon cancer
229 cells was obtained from *HT* asparagus. Of note, we have previously published
230 that these saponins are resistant to simulated digestion [19] and due to their
231 poor intestinal absorption [36], they are colon-accessible, where they may exert
232 their biological effects. This represent an advantage respect to *5-FU* that suffers
233 a reduction of cellular drug uptake because water-soluble drugs may attach to
234 transporters carrying nutrients and therefore fail to accumulate in the colonic
235 tumoral cells at relevant concentrations [7, 37-38].

236 We have previously demonstrated that the total extract of *HT* asparagus
237 interfered with MEK, mTOR and PI3K pathways by inhibiting the
238 phosphorylation of ERK, p70S6K, and AKT proteins HCT-116 cells which
239 presented a dietary strategy against colon cancer cells [19]. Indeed, dual
240 targeted inhibition of MEK and PI3K pathway effectors presents a potential
241 strategy to overcome resistance to MEK inhibitor therapy [39]. HCT-116, Caco-
242 2 and HT-29 cells have a different mutation profile. In HCT-116 cells, *KRAS* and
243 *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 a 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 on extraction with aqueous ethanol, purification by adsorption chromatography with Amberlite XAD-16 resin, separation by reverse 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 effect 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 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 due to *HT* genotypes could be consider a new source of possible agents for treatment of chemo-resistant tumors.

Experimental section

Chemicals and Reagents.

PD (97% purity checked by NMR) was purchased from Chromadex Chemical Co. (Barcelona, Spain). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-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 12000 rpm 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 reverse-phase analytical column (25 cm length x 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 on-line 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 semipreparative SYNERGI 4 μ m HYDRO-RP 80A reverse-phase column (25 cm length x 21.20 mm i.d., 4 μ m 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 μ L. 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, centrifuged at 12000 rpm 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 5A medium supplemented with 10% heat-inactivated fetal bovine serum (*FBS*), 100 U/mL penicillin, and 172 μ M streptomycin; Caco-2: Dulbecco's modified Eagle essential medium (DMEM), 10% *FBS*, 1% non-essential amino acids, 100 U/mL penicillin, and 172 μ M streptomycin. All cell lines were cultured at 37 °C and 5% CO₂ 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 10⁴ cells/well in 200 μ L 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 μ M) 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 μ L 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% CO₂. The supernatant was then sucked off, after which 100 μ L of DMSO was 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, each experiment was including 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.

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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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Figure captions

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

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 μ M) of HSAP-1, HSAP-3, HSAP-6, HSAP-8, HSAP10, PD or 5-FU for 48 h. All assays were carried out in three separate trials, each experiment was including five replicate sets. Data was normalized to control cells (set at 100%) and shown as mean $\pm$ SD.

Figure 3.- Chemical structure of protodioscin

554 **Table 1:** Saponins identified by HPLC-MS (+ / -) of triguero *HT* asparagus. Each compound was isolated and the ion fragmentation was
555 analyzed three times (n=3).

Saponin	Rt (min)	MW	Molecular ion (m z ⁻¹)		Ion fragmentation	
			Negative ion	Positive ion	Negative mode	Positive mode
<i>HTSAP-1</i>	21	1052	1051 [M-H] ⁻	1075 [M+Na] ⁺	- <i>Pen</i> 919- <i>Hex</i> 757- <i>Hex</i> 595- <i>Hex</i> 433*	[-Na-H ₂ O]1035- <i>Hex</i> 873- <i>Hex</i> 711- <i>Pen</i> 579- <i>Hex</i> 417
<i>HTSAP-3</i>	25	1022	1021 [M-H] ⁻	1045 [M+Na] ⁺	- <i>Pen</i> 889- <i>Pen</i> 757- <i>Hex</i> 595- <i>Hex</i> 433*	[-Na-H ₂ O]1005- <i>Pen</i> 873- <i>Hex</i> 711- <i>Pen</i> 579- <i>Hex</i> 417
<i>PD</i>	27	1048	1047 [M-H] ⁻	1071 [M+Na] ⁺	- <i>DoHex</i> 901- <i>DoHex</i> 755- <i>Hex</i> 593- <i>Hex</i> 431*	[-Na-H ₂ O]1031- <i>Hex</i> 869- <i>DoHex</i> 723- <i>DoHex</i> 577- <i>Hex</i> 415
<i>HTSAP-6</i>	28	1036	1035 [M-H] ⁻	1059 [M+Na] ⁺	- <i>Pen</i> 903- <i>DoHex</i> 757- <i>Hex</i> 595- <i>Hex</i> 433*	[-Na-H ₂ O]1019- <i>DoHex</i> 873- <i>Pen</i> 741- <i>Hex</i> 579- <i>Hex</i> 417
<i>HTSAP-8</i>	30	904	903 [M-H] ⁻	927 [M+Na] ⁺	- <i>DoHex</i> 757- <i>Hex</i> 595- <i>Hex</i> 433*	[-Na-H ₂ O]887- <i>Hex</i> 725- <i>DoHex</i> 579- <i>Hex</i> 417
<i>HTSAP-10</i>	27	1048	1047 [M-H] ⁻	1071 [M+Na] ⁺	- <i>DoHex</i> 901- <i>DoHex</i> 755- <i>Unk</i> 595- <i>Hex</i> 433*	[-Na-H ₂ O]1031- <i>Hex</i> 869- <i>DoHex</i> 723- <i>DoHex</i> 577- <i>Unk</i> 417

556 *Rt*: retention time, *MW*: molecular weight; * *Genin*; *Pen*: Pentose; *DoHex*: Deoxihexosa; *Hex*: Hexose; *Unk*: Unknown

Figure 1

Figure 2

Figure 3