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Article

# Cytotoxicity and Chemotaxonomic Significance of Saponins from Wild and Cultured *Asparagus* Shoots

Tarik Chileh-Chelh<sup>1</sup>, Rosalía López-Ruiz<sup>2</sup>, Ana M. García-Cervantes<sup>3</sup>, Ignacio Rodríguez-García<sup>3</sup>, Miguel A. Rincón-Cervera<sup>1,4</sup>, Mohamed Ezzaitouni<sup>1</sup> and José L. Guil-Guerrero<sup>1,\*</sup>

<sup>1</sup> Food Technology Division, University of Almería, 04120-Almería, Spain

<sup>2</sup> Dpto. Química-Física, Química Analítica de Contaminantes, Universidad de Almería, 04120-Almería, Spain

<sup>3</sup> Dpto. Química-Física, CIAIMBITAL, Universidad de Almería, 04120-Almería, Spain

<sup>4</sup> Institute of Nutrition and Food Technology, University of Chile. 7830490-Santiago, Chile

\* Correspondence: jlguil@ual.es; Tel.: +34640225578

**Abstract:** *Asparagus* shoots are worldwide consumed, although most of such species have a restricted range, and several taxa remain unstudied. In this work, a total of four taxa from different locations were scrutinized and compared with cultivated *A. officinalis*. All shoots were screened for saponins by LC-MS, and *in vitro* antiproliferative activities against the HT-29 colorectal cancer cell line (by MTT assay). The total saponins (TS) contained in the crude extracts ranged from 710.0 (*A. officinalis*) to 1258.6 mg/100 g dw (*A. acutifolius*). It stands out the richness of compounds detected in this work: a total of 47 saponins have been detected and quantified in the edible parts (shoots) of 5 taxa of *Asparagus*. The structure of all the saponins found present skeletons of the furostane and spirostane type. In turn, the structures with a furostane skeleton are divided into unsaturated and dioxygenated, both in the 20–22 position. The sum of dioscin and derivatives varied largely along the studied taxa. It reached the following percentages of TS: 27.11 (*A. officinalis*), 18.96 (*A. aphyllus*), 5.37 (*A. acutifolius*), and 0.59 (*A. albus*), while in *A. horridus* such compound remains undetected. Aspachioside A, D, and M varied largely among samples, while a total of seven aspasirostanosides were characterized in the analyzed species. Hierarchical cluster analysis of the saponin profiles clearly separated the various taxa and demonstrated that the taxonomic position is more important than the place from which the samples were acquired. Thus, saponin profiles have chemotaxonomic significance in *Asparagus* taxa. The MTT assay showed dose- and time-dependent inhibitory effects of several saponins extracts on HT-29 cancer cells, standing out the cell growth inhibition exercised by *A. albus* and *A. acutifolius* (GI<sub>50</sub> of 125 and 175 µg/mL). This work constitutes a whole approach to evaluate the saponins from shoots of different *Asparagus* taxa and provide arguments for use them as functional foods.

**Keywords:** *Asparagus*; saponins; LC-MS; HT-29 cells; MTT

## 1. Introduction

Wild edible plants (WEPs) have been an essential component of human diets across cultures and continents for centuries. These plants offer a diverse array of essential nutrients, including vitamins, minerals, dietary fibre, micronutrients, and phytochemicals. In fact, they are often rich in antioxidants, and therefore, valuable contributors to human nutrition (Johns and Eyzaguirre, 2006). Their incorporation into diets can enhance nutritional diversity and address deficiencies of conventional diets, promoting better health outcomes.

The use of WEPs is deeply rooted in cultural traditions and practices. Indigenous and local communities have developed extensive knowledge about the identification, harvesting, and preparation of these plants, passing this wisdom down through generations. The food use of WEPs reflects cultural heritage, connects people to their natural surroundings, and strengthens cultural

identity (Pieroni and Price, 2006). Moreover, WEPs serve as a valuable safety net during times of food scarcity and play a crucial role in enhancing food security. These plants are often resilient to environmental stressors and can thrive in diverse ecosystems, providing a reliable source of nutrition, especially in regions with limited access to conventional crops. Then, incorporating WEPs into agricultural systems can contribute to sustainable farming practices. Many of these plants require minimal inputs and are well-suited for organic and agroecological approaches. Their cultivation can enhance biodiversity, reduce the environmental impact of agriculture, and foster resilient food systems (Heywood and Dulloo, 2006; Kuhnlein et al., 2013).

*Asparagus* is a genus in the Liliaceae family that includes over 250 species, from which *Asparagus officinalis* L. is the only cultivated species. However, there are several wild species that traditionally are collected for consumption and medicinal purposes in the Mediterranean Basin, such as *A. acutifolius* L. and *A. horridus* L. (syn. *A. stipularis* Forssk) (Adouni et al., 2018). *A. officinalis* is a widely cultivated vegetable with a long history of use as both food and medicine. While the saponins content of the roots of *A. officinalis* have been extensively studied, the shoots of this plant have also been found to contain significant amounts of saponins. These are structurally similar to those found in the roots of this plant. Such compounds are integrated by a triterpenoid aglycone, as protodioscin or dioscin, which are attached to one or more sugar residues. The sugar moieties are typically glucose, rhamnose, or xylose, and are linked to the aglycone through an ether bond. However, the exact composition of the saponins in *Asparagus* shoots can vary depending on the variety of the plant and the conditions under which it is grown (Zhang et al., 2015).

For instance, Lee et al. (2000) reported protodioscin (1.4–5 mg/100 g fw) in the white shoots of *A. officinalis*, while Wang et al. (2003) reported protodioscin at 0.024–2.5 g/100 g fw in green *A. officinalis*. Concerning *A. acutifolius*, the saponins HTSAP-1, -3, -6, and ACSAP-1 were reported (Hamdi et al., 2021).

The saponins present in *Asparagus* shoots exhibit a wide range of biological activities, including anti-inflammatory, antioxidant, antitumor, and immunomodulatory properties. However, further research is needed to fully understand the mechanisms by which these compounds exert their effects and to determine the optimal dosages and administration methods for their use (Ohtsuki, et al., 2007; Han et al., 2012; Choi et al., 2012).

Studies on the saponins of *A. acutifolius* shoots suggest that these compounds may have several potential health benefits, being similar to the saponins of *A. officinalis*. For example, they may have anti-inflammatory, antioxidant, antitumor, and immunomodulatory properties. Some studies have also suggested that these compounds may have a role in promoting healthy digestion and regulating blood lipid levels (Han et al., 2012).

Wild *Asparagus* shoots are a rich source of saponins, which have been found to possess a range of biological activities and potential health benefits. Although the phytochemistry and phytotherapy of cultivated *A. officinalis* and wild *A. acutifolius* are relatively well known, there is a lack of knowledge on the saponins and biological activities of *A. stipularis*, *A. albus*, and *A. aphyllus* young shoots. Moreover, the variability of saponins composition of the various *Asparagus* taxa depending on their ecogeographic location constitute unexplored research. Therefore, this work was designed to determine saponins profiles of several relevant edible shoots of this genus collected in South Spain, as well as their antitumor activities, seeking to unravel their health benefits.

## 2. Results

### 2.1. Moisture Content

Considering all samples, moisture content ranged from 81.4 in *A. acutifolius* AC1 to 91.1 g/100 g in *A. officinalis* O1. Focusing on mean values of species, such amount was between 84.6 (*A. horridus*) and 91.0 g/100 g (*A. officinalis*).

## 2.2. Total Saponins and Saponin Profiles

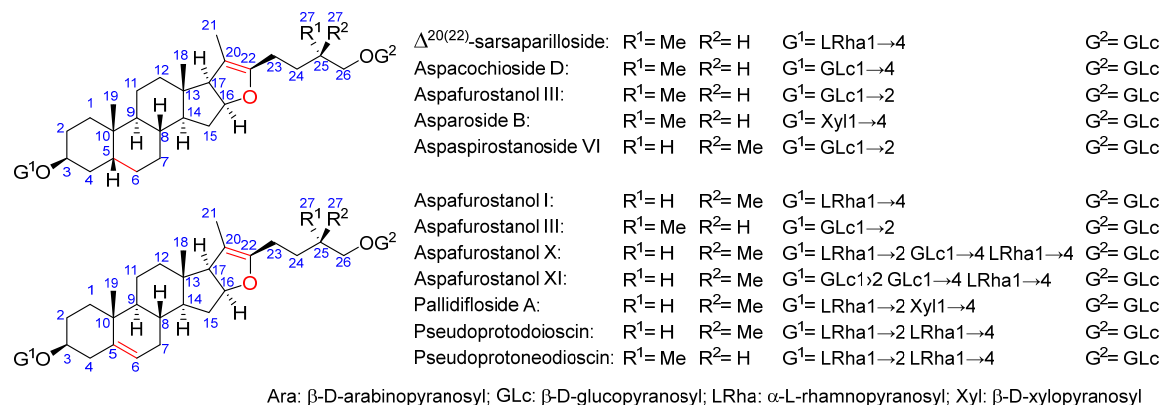
Total saponins (Table 1) ranged in samples from 669.1 (*A. officinalis* O2) to 1529.3 mg/100 g dw (*A. acutifolius* AC2). As for species, values were from 710.0 (*A. officinalis*) to 1258.6 mg/100 g dw (*A. acutifolius*). Considering fresh weight, amounts ranged from 63.9 (*A. officinalis*) to 157.2 mg/100 g (*A. horridus*).

**Table 1.** Moisture and saponins content of *Asparagus* samples <sup>a</sup>.

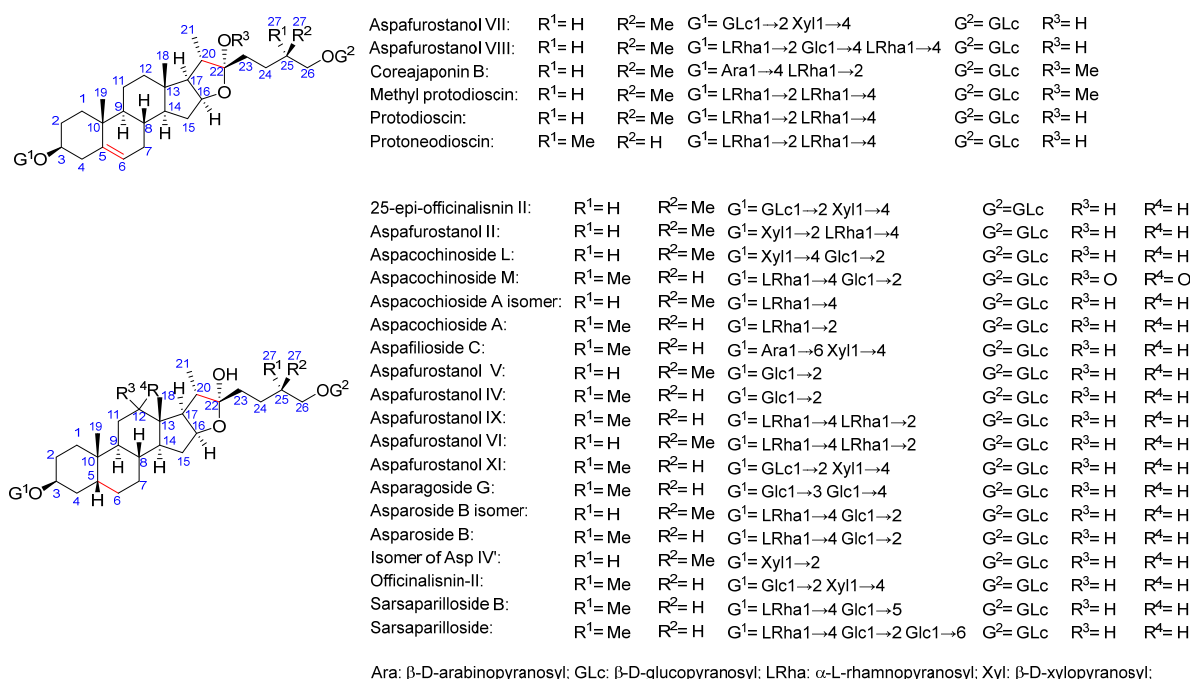
Species/samples	Moisture (g/100 g)	Saponins (mg/100 g dry weight)
<i>A. acutifolius</i>		
AC1	81.4 ± 0.0 <sup>h</sup>	1094.9 ± 21.2 <sup>def</sup>
AC2	84.6 ± 0.3 <sup>fg</sup>	1529.3 ± 107.8 <sup>a</sup>
AC3	85.2 ± 0.3 <sup>ef</sup>	1151.5 ± 7.9 <sup>de</sup>
Mean ± SD	84.5 ± 2.3 <sup>B</sup>	1258.6 ± 236.2 <sup>A</sup>
<i>A. albus</i>		
AL1	88.0 ± 0.2 <sup>bc</sup>	1405.2 ± 176.3 <sup>ab</sup>
AL2	88.1 ± 0.2 <sup>bc</sup>	996.9 ± 39.1 <sup>efg</sup>
AL3	86.4 ± 1.2 <sup>de</sup>	1387.4 ± 107.9 <sup>abc</sup>
AL4	89.0 ± 0.5 <sup>b</sup>	930.5 ± 98.5 <sup>fgh</sup>
Mean ± SD	87.9 ± 1.1 <sup>AB</sup>	1180.0 ± 251.3 <sup>AB</sup>
<i>A. aphyllus</i>		
AP1	83.7 ± 0.9 <sup>g</sup>	907.8 ± 27.3 <sup>fgh</sup>
AP2	85.3 ± 0.5 <sup>ef</sup>	860.8 ± 115.0 <sup>gh</sup>
AP3	86.5 ± 1.2 <sup>de</sup>	909.3 ± 7.7 <sup>fgh</sup>
Mean ± SD	85.2 ± 1.4 <sup>B</sup>	892.6 ± 27.6 <sup>AB</sup>
<i>A. horridus</i>		
H1	80.7 ± 1.4 <sup>h</sup>	1211.5 ± 60.3 <sup>cd</sup>
H2	84.0 ± 0.7 <sup>fg</sup>	1422.9 ± 70.8 <sup>ab</sup>
H3	83.3 ± 1.1 <sup>g</sup>	838.3 ± 174.1 <sup>ghi</sup>
H4	87.5 ± 0.1 <sup>cd</sup>	838.1 ± 55.5 <sup>ghi</sup>
H5	87.3 ± 0.5 <sup>cd</sup>	794.9 ± 31.2 <sup>hi</sup>
Mean ± SD	84.6 ± 2.9 <sup>B</sup>	1021.1 ± 281.0 <sup>AB</sup>
<i>A. officinalis</i>		
O1	91.1 ± 0.2 <sup>a</sup>	750.9 ± 112.0 <sup>hi</sup>
O2	90.9 ± 0.3 <sup>a</sup>	669.1 ± 101.0 <sup>i</sup>
Mean ± SD	91.0 ± 0.1 <sup>A</sup>	710.0 ± 57.8 <sup>B</sup>

<sup>a</sup> Data represent means ± SD of samples analyzed in triplicate. Differences in saponin amounts were tested according to one-way ANOVA followed by Duncan's test. Within a file, means followed by different lowercase letters are significantly different at  $P < 0.05$ , and means followed by capital letters represent the ANOVA test effected for mean values of species ( $P < 0.05$ ).

As for saponin profiles (Supplemental Table 1), a total of 47 different compounds were detected among the various samples. The structure of all the saponins found present skeletons of the furostane and spirostane type. In turn, the structures with a furostane skeleton are divided into unsaturated and dioxygenated, both in the 20–22 position (Figures 1, 2 and 3). All the structures are detailed in Supplemental Table 2.



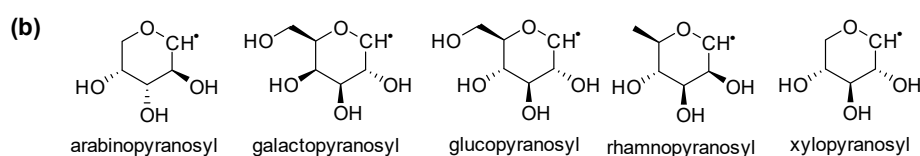
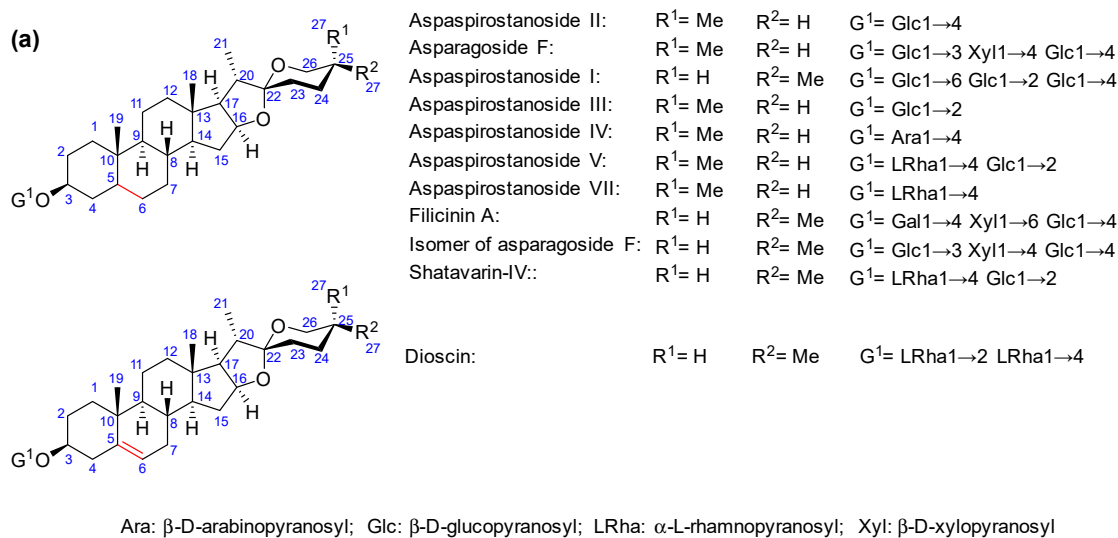
**Figure 1.** Asparagus saponins with furostane structure with an insaturation in C20-C22.



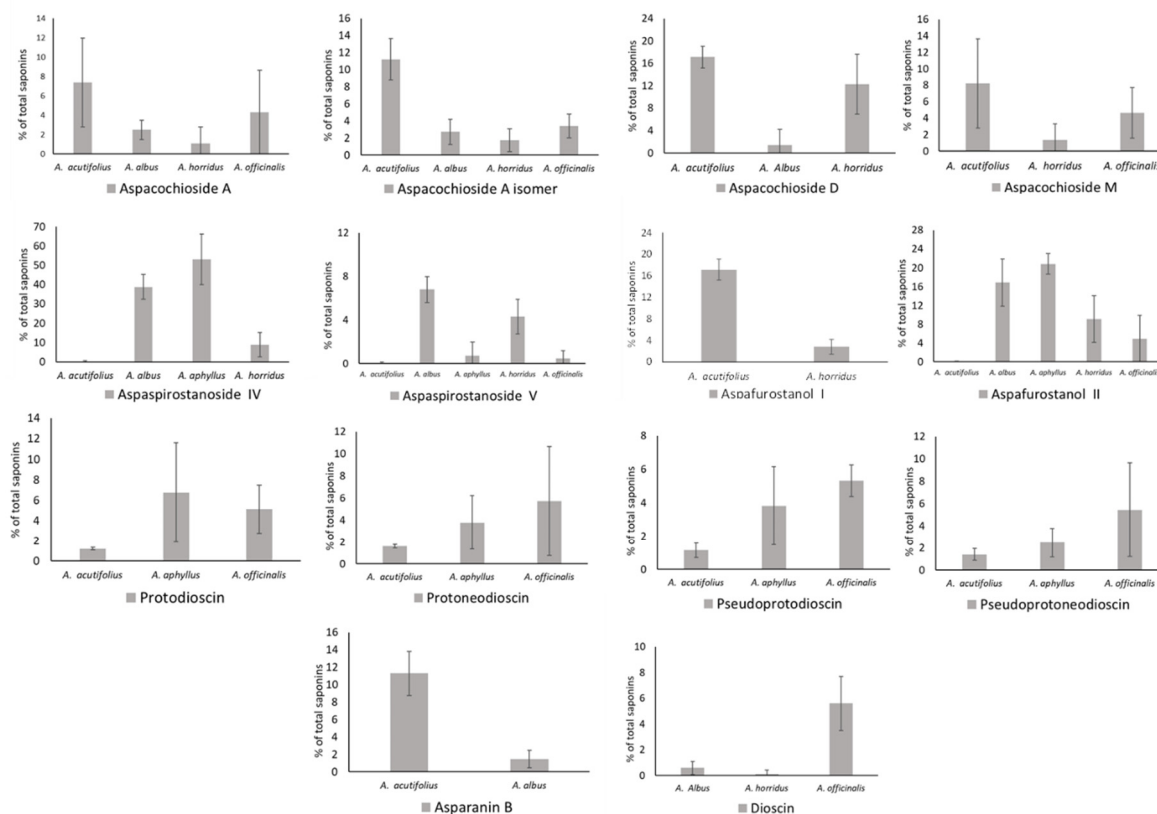
**Figure 2.** Asparagus saponins with furostane structure dioxxygenated at C22.

The occurrence of all these molecules is detailed in Supplemental Table 1, while the amounts of the main saponins are depicted in Figure 4. Aspachioside A and Aspachioside A isomer were absent in *A. aphyllus*, and *A. acutifolius* showed the highest values, 7.37 and 11.24% of total saponins (TS). Aspachioside D was present in *A. acutifolius* (17.12), *A. albus* (1.40), and *A. horridus* (12.33% TS), and Aspachioside M occurs in *A. acutifolius* (8.13), *A. horridus* (1.32), and *A. officinalis* (6.79% TS). Dioscin was found in *A. albus*, *A. horridus*, and *A. officinalis*, reaching significant values only in the latter, 5.61% TS. Dioscin derivatives (protodioscin, protoneodioscin, pseudoprotodioscin, and pseudoprotoneodioscin) were detected in *A. acutifolius*, *A. aphyllus*, and *A. officinalis*. Protodioscin highlights in *A. aphyllus* (6.73) and *A. officinalis* (5.11% TS); protoneodioscin ranged from 1.66 (*A. acutifolius*) to 5.72% TS (*A. officinalis*); pseudoprotodioscin was between 1.14 (*A. acutifolius*) and 4.56% TS (*A. officinalis*); and pseudoprotoneodioscin ranged from 1.41 (*A. acutifolius*) to 5.44% TS (*A. officinalis*). Asparanin B was detected only in *A. acutifolius* and *A. albus*, at 11.30 and 1.49% TS. A total of seven aspastrostanosides were characterized in the analyzed species. From these, it reached noticeable amounts the aspastrostanoside IV, which was absent in *A. officinalis*, and ranged from 0.22 (*A. acutifolius*) to 53.03% TS (*A. aphyllus*), and aspastrostanoside V, which occurs in all species, ranging from 0.05 (*A. acutifolius*) and 6.81% TS (*A. albus*). As for aspafurostanols, the I one was present in *A. acutifolius* (17.14) and *A. horridus* (2.83% TS), while the II one was found in all species, ranging from 0.04 in *A. acutifolius* to 20.84 in *A. aphyllus*. Some saponins were restricted to

one single species; for instance, filicin A was detected in *A. horridus* (11.80%), while aspafulurostanols VIII-XI were restricted to *A. officinalis*, ranging from 5.31 (aspafulurostanol VIII) to 10.43% TS (aspafulurostanol XI).



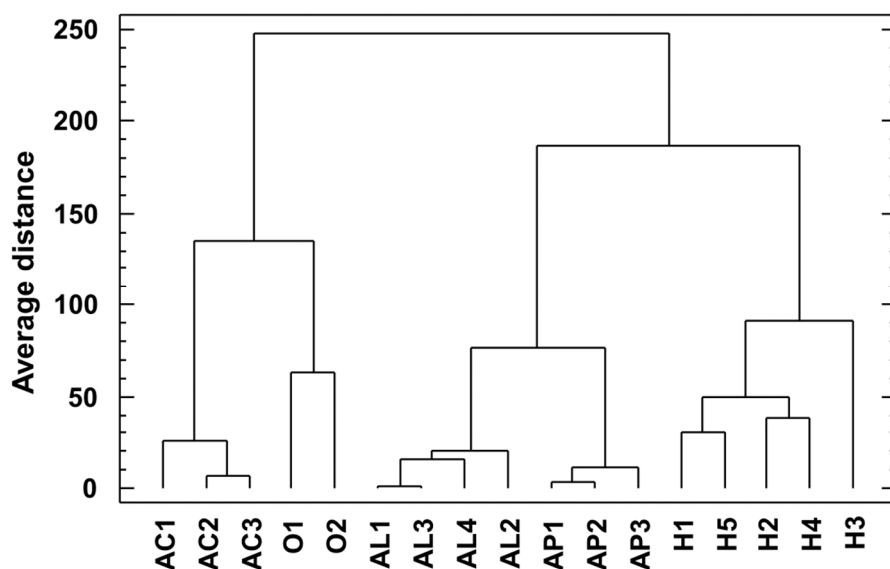
**Figure 3. (a)** Asparagus saponins with spirostane structure. **(b)** Common sugars in all structures.



**Figure 4.** Occurrence of the main saponins detected in Asparagus shoots.

### 2.3. Cluster Analysis

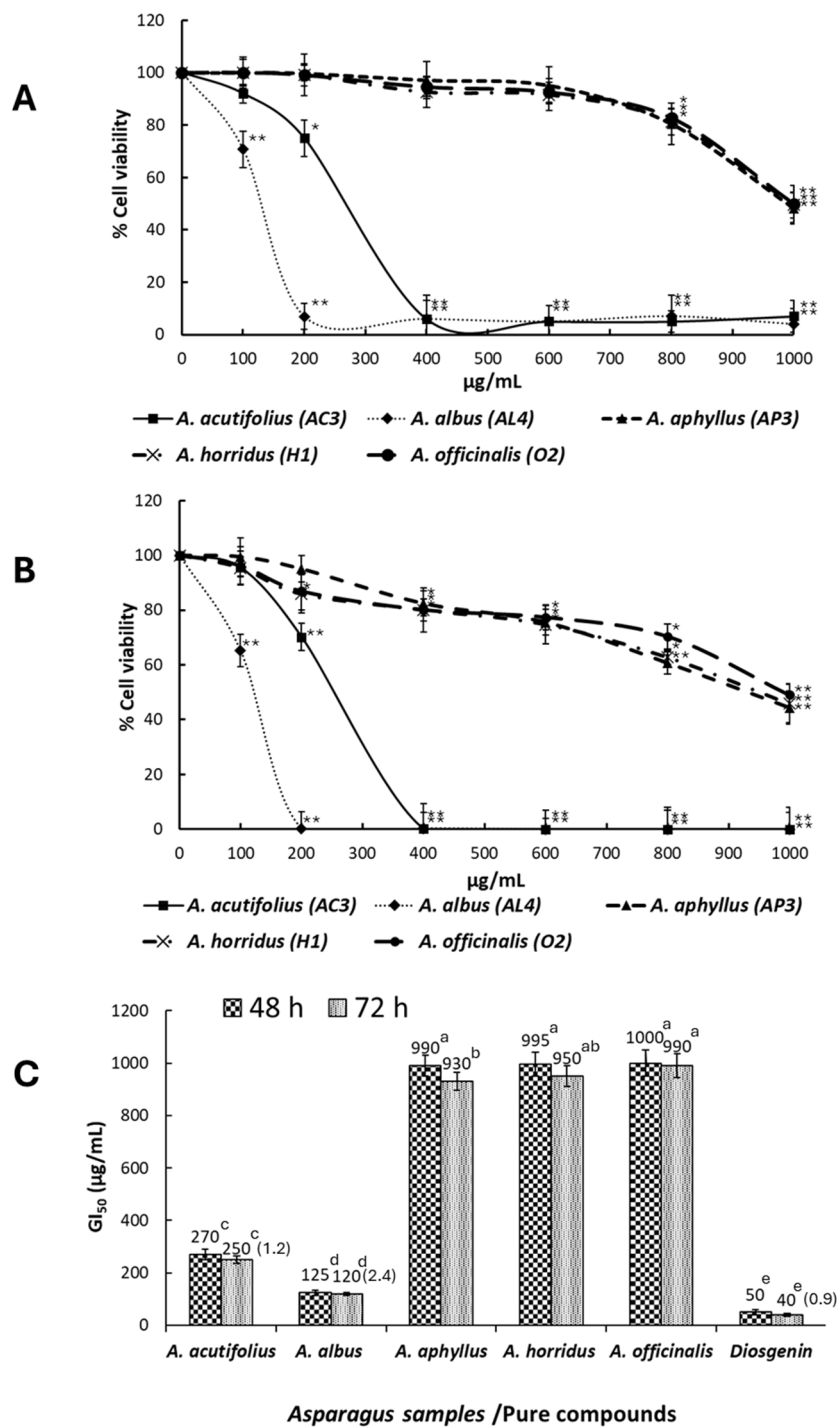
Figure 5 shows the Dendrogram obtained from a cluster analysis of all detected saponin profiles of sampled *Asparagus* species. Samples were clustered using Ward's technique based on city block distance measure.



**Figure 5.** Dendrogram obtained from a cluster analysis of saponin profiles of sampled *Asparagus* species. Samples were clustered using Ward's technique based on city-block distance measure.

### 2.4. Antiproliferative Activity

The MTT assay was accomplished to evaluate the inhibitory effects of extracts-containing *Asparagus* saponins on HT-29 human colorectal cancer cell viability. Extracts having the highest antioxidant activity (one of each species) were selected for this assay. Figures 6A and 6B show the activity of such extracts against HT-29 cancer cells viability after 48 and 72 h of treatment. Cell growth inhibition was exercised much better by extracts from *A. albus* and *A. acutifolius*, which at 400  $\mu\text{g}/\text{mL}$  and after 48 h of cell exposure to extracts induced 6.9 and 92.0% of cell viability, and at 72 h of cell exposure induced 0.3 and 80.7% of cancer cell viability in comparison with controls without extract addition.  $\text{GI}_{50}$  values, i.e., the doses of extracts that inhibited cell growth by 50%, of all samples are shown in Figure 6C. After a 72-h incubation period,  $\text{GI}_{50}$  for *A. acutifolius* (AC3), *A. albus* (AL4), *A. aphyllus* (AP3), *A. horridus* (H1), and *A. officinalis* (O2), were 250, 120, 930, 950, and 999  $\mu\text{g}/\text{mL}$ , respectively.  $\text{GI}_{50}$  for diosgenin after 48 and 72 h of cells exposure to extracts were 50 and 40  $\mu\text{g}/\text{mL}$ . The SI of HT-29 cancer cells versus CCD-18 normal cells was evaluated at 72 h of cells exposure to extracts having  $\text{GI}_{50} \leq 250$   $\mu\text{g}/\text{mL}$ , and it was 1.2 (*A. acutifolius*) to 2.4 (*A. albus*), while for diosgenin it was 0.9.



**Figure 6.** MTT assay. 6A) Dose-response curves of HT-29 cell viability after treatment with different concentrations of saponin extracts of Asparagus samples for 48 h. 6B) Dose-response curves of HT-29 cell viability after treatment with different concentrations of saponin extracts of Asparagus samples for 72 h. The statistical significance was evaluated at  $P < 0.05$  (\*) and  $P < 0.001$  (\*\*). 6C)  $\text{GI}_{50}$  of HT-29 cells after cells treatment with saponin extracts of Asparagus samples and diosgenin for 48 and 72 h. The  $\text{GI}_{50}$  value is detailed over columns, and the Selectivity Index (SI) for 72-h exposed (HT-29 vs. CCD-18) cells to saponin extracts is shown in parentheses. Data represent the mean of three complete independent experiments  $\pm$  SD (error bars). In a bar, means followed by different letters are significantly different at  $P < 0.05$ .

### 3. Discussion

#### 3.1. Saponin Profiles of the Various *Asparagus* Species Analyzed in This Work

The total saponins-contained crude extract ranged from 710.0 (*A. officinalis*) to 1258.6 mg/100 g dw (*A. acutifolius*). These results are in line with those of Shao et al. (1996), who obtained 1.72 g/100 g dw from *A. officinalis* shoots (gravimetrically determined). Vázquez-Castilla et al. (2013) reported very low amounts in shoots: 10.9–27.3 mg/kg fw of Huétor *Asparagus*. Concerning *A. acutifolius*, Hamdi et al. (2021) reported total saponins at 1419 mg/kg dw.

*Asparagus* saponins are steroidal glycosides. In *A. officinalis*, and most of the green and white commercial hybrids derived from this specie, the main saponin is protodioscin (C<sub>51</sub>H<sub>84</sub>O<sub>22</sub>), which is a glycoside derivative of the furostanoid type diosgenin (Vázquez-Castilla et al., 2013). To date, about more than 20 saponin aglycones have been identified in the genus *Asparagus*, however, only sarsasapogenin, asparanin A, protodioscin, yamogenin, and its derivatives have been studied (Pegiou et al., 2020). Then, the richness of compounds detected in this work stands out. A total of 47 saponins have been detected and quantified in the edible parts (shoots) of 5 taxa of wild *Asparagus* and farmed *A. officinalis*.

The sum of dioscin and derivatives, i.e., protodioscin, protoneodioscin, pseudoprotodioscin, pseudoprotoneodioscin, and methyl protodioscin, varied largely along the studied taxa. It reached the following percentages of TS: 27.11 (*A. officinalis*), 18.96 (*A. aphyllus*), 5.37 (*A. acutifolius*), and 0.59 (*A. albus*), while in *A. horridus* such compound were undetected. Interestingly, diosgenin, a protodioscin moiety, was not found.

#### 3.2. Multivariable Analyses for Assessing Chemotaxonomy

Figure 2 shows the Dendrogram obtained from a cluster analysis of saponin profiles of sampled *Asparagus* species, where these are clearly separated. From these results, it is evident that the taxonomic position is more important than the place from which the samples were acquired. *Asparagus* is a complex genus in which has been reported a notable disagreement between molecular phylogeny and morphological taxonomy; for instance, several species belong to larger species complexes, both paraphyletic and polyphyletic ones. Thus, species delimitation should be based on both molecular and morphological data (Norup et al., 2015).

Four species analyzed belong to *Asparagus* subgenus: *A. acutifolius*, *A. aphyllus*, *A. horridus*, and *A. officinalis*, while *A. albus* belongs to subgenus *Asparagopsis*. In the obtained dendrogram, a close position was obtained for *A. horridus* and *A. aphyllus*, which were previously typified as genetically related (Norup et al., 2015). However, *A. albus* was close to *A. acutifolius*, although a clear relationship between both species has not been reported yet. Probably, this fact was due to the absence of other members of the *Asparagopsis* subgenus in this analysis, and this fact is worthy of further research before confirming the utility of *Asparagus* saponins as chemotaxonomical tool for subgenus *Asparagus* classification.

#### 3.3. Antiproliferative Activity of the Saponins Extracts of *Asparagus* Shoots on HT-29 Cancer Cells

The saponins from *Asparagus* spp. have long been characterized as having antitumor activity. For instance, the crude saponins extract from the shoots of *A. officinalis* were cytostatic and cytotoxic against the human leukemia HL-60 cells, and inhibited the synthesis of DNA, RNA, and proteins (Shao et al., 1996).

Reports indicated that the cytotoxic activity is characteristic of each *Asparagus* organ. Overall, the ethanolic extracts of rhizome and leaf are cytotoxic; however, low activity has been described for shoot extracts (Hamdi et al., 2021). The rhizome extracts from several *Asparagus* species were tested against the HepG2 (liver cancer) cell line. Three *Asparagus* species, namely *A. acutifolius* (Hamdi et al., 2021), *A. adscendent* (Khan et al., 2017), and *A. filicinii* (Liu et al., 2015) exercised noticeable cytotoxic activity, and this activity has been related to the occurrence of saponins and their genins. However, the rhizome extract of *A. albus* showed low activity (Hamdi et al., 2017).

Some pure saponins isolated from *Asparagus* spp. have been tested against cancer cells. For instance, asparanin A, a steroidal saponin, exhibited anticancer activity on endometrial cancer. This saponin inhibited cell proliferation and caused cell morphology alteration and cell cycle arrest in G0/G1 phase, the apoptosis through mitochondrial pathway, generation of ROS, and activation of caspases, besides other mechanisms. In vivo inhibited the tumor cell proliferation and growth, and induced apoptosis (Zhang et al., 2020). Asparanin A also induces cell cycle arrest and triggers apoptosis via a p53-independent manner in HepG2 cells (Liu et al., 2009).

In terms of activity against colorectal cancer cells, the saponins from *Asparagus* have been typified as inhibitors through cytotoxicity and apoptosis (Bousserouel et al., 2013). For instance, the saponins from edible spears of wild asparagus (triguero Huétor-Tájar, HT, landrace) inhibit AKT, p70S6K, and ERK signaling, and induce apoptosis through G0/G1 cell cycle arrest in human colon cancer HCT-116 cells (Jaramillo et al., 2016). Both the rhizome and leaf from *A. acutifolius* showed high activity against this cell line, while the leaf extracts from *A. officinalis* and *A. acutifolius* species had similar IC<sub>50</sub> values (Hamdi et al., 2017). Interestingly, when checking the rhizome extract of *A. officinalis* against HCT-116 cells, the IC<sub>50</sub> value was better than that of the saponins extracted from the corresponding by-products (Wang et al. 2013), and this result was related to a different saponin composition or to the synergistic effects among the various phytochemicals presents in *A. acutifolius* extracts.

Zhao (2012) reported activity of the saponins-containing crude extract against the colon cancer cell lines SW620 and HCT-116 through induction of cytotoxicity. Jaramillo-Carmona et al. (2018) found that protodioscin induced cytotoxicity in HCT-116, HT-29, and Caco-2 colon cancer cells. Dioscin exercises antitumor activities against several types of tumors, such as lung cancer, gastric cancer, colon cancer, glioblastoma, cervix carcinoma, ovarian cancer, breast cancer, prostate cancer, and leukemia. Its antitumor activity is exercised through intrinsic mitochondrial apoptosis, involving activation of caspase-9 and caspase-3, and induces a reduction in antiapoptotic proteins such as Bcl-2, Bcl-xl, cIAP-1, and Mcl-1 (Yang et al., 2019). Kang et al. (2011) checked the activity of asparanin A against colon cancer HCT-15 cells and found that this compound induced apoptosis and inhibited cells proliferation through cell-cycle arrest in the G0/G1 and G2/M phase.

In this work, after 48 and 72 h of treatment, the MTT assay revealed concentration- and time-dependent inhibitory effects on HT-29 cells for all assayed extracts (Figures 6A and 6B). The antitumor activity was especially intense for the extracts obtained from the stems of *A. albus* and *A. acutifolius*. In the case of *A. albus*, it contains saponins that in descending order are aspasirostanoside IV, aspafurostanol II, and aspasirostanoside V. However, considering that these same saponins are found in *A. aphyllus*, which develops low activity against HT-29 cells, it is difficult to attribute the observed activity to such saponins. This is not the case of *A. acutifolius*, which contains characteristic saponins, such as aspafurostanol I, asparanin B (shatavarin-IV), and aspachioiside M, which could have exerted the noted action. Interestingly, these two highly active species are the only ones that contain shatavarin IV, especially *A. acutifolius*. This saponin was previously isolated from *A. racemosus* roots. The cytotoxicity (in vitro) of shatavarin IV extracts (approximately 5% of shavaratins) and other shatavarins rich fraction was assayed by the MTT test against HT-29 cells, showing significant anticancer activity in both in vitro and in vivo experimental models (Mitra et al., 2012). Therefore, considering the content of shavaratin IV (11.30 in *A. acutifolius* and 1.49% in *A. albus*), it is likely that the noted activity was due to this saponin type, at least partially.

Although *A. officinalis* shows high percentages of dioscin and its derivatives, the activity of its crude extract against HT-29 cells was very week, which induces to consider that this cell line is low sensitive to these saponin types.

The National Cancer Institute (NCI) consider that compounds/extracts/fractions as cytotoxic when the GI<sub>50</sub> values are within the 20–30 µg/mL range (Boik, 2001). Based on the MTT results, the saponin extracts checked lack cytotoxicity, while diosgenin was recognized as cytotoxic to the tested cell line. It should be noted that the extracts tested are not completely made up of saponins; therefore, it is quite possible that isolation of pure saponin fractions from these extracts will yield cytotoxic compounds. The GI<sub>50</sub> value of diosgenin for the HT-29 cell line obtained in the current work by the

MTT assay is consistent with previous studies for such compound on HeLa cancer cell line (e.g., Stefanowicz-Hajduk et al., 2021). On the other hand, according to the threshold proposed by Suffness and Pezzuto (1990), crude extracts showing a  $GI_{50} \leq 100 \mu\text{g/mL}$  can be selected for further studies, whereas the most promising ones are those with a  $GI_{50} < 30 \mu\text{g/mL}$ . Thus, the saponin extract from *A. albus* shoot, whose  $GI_{50}$  is close to this figure ( $120 \mu\text{g/mL}$ ), merit further research for its fractionation until pure active compounds are isolated. Then, the mechanisms of action of such compounds against different cancer cell lines would be checked according to different methodologies.

It should be noted that the antiproliferative effects of the extracts may not be due to specific compounds. It is likely that interactions among the various saponins and with other components of the extract could have contributed to the overall reported effects. In this regard, it has been reported the anticancer effects of the deproteinized *Asparagus* polysaccharide on hepatocellular carcinoma cells. *Asparagus* polysaccharide exercised effective inhibitor effects on cell growth in vitro and in vivo and exert potent selective cytotoxicity against human hepatocellular carcinoma Hep3B and HepG2 cells. Such polysaccharide develops activity through an apoptosis-associated pathway by modulating the expression of Bax, Bcl-2, and caspase-3, and has been proposed as a potential therapeutic agent (or chemosensitizer) for liver cancer therapy (Xiang et al., 2014).

## 4. Materials and Methods

### 4.1. Samples

Shoots were collected in the locations listed in Table 2 or purchased in local markets. Upon arrival to the laboratory, the shoots were labeled, weighed, measured, and placed in a glass desiccator until analysis. Just prior to analysis, shoots were ground to powder with a mortar. Approximately 2 g of each sample was used for moisture analysis, which was carried out in a forced air oven at  $105^\circ\text{C}$  for 8 h. All results are reported on a dry weight (dw) basis.

**Table 2.** Data on samples collection of the shoots *Asparagus* species.

Species/Location	Code	Geographical coordinates	Date
<i>Asparagus acutifolius</i> (Raviscanina)			
Punta Entinas, El Ejido, Almería	AC1	36.690831 -2.7732501	04/04/2023
Algámitas, Sevilla	AC2	37.018445 -5.161181	07/03/2023
Fonelas, Granada	AC3	37.409918 -3.200831	10/05/2023
<i>Asparagus albus</i> (White asparagus)			
Darrical, Almería	AL1	36.917674 -3.028230	17/03/2023
Sierra Cabrera, Almería	AL2	37.134984 -1.868005	05/01/2023
Barranco de las Lastras, Adra, Almería	AL3	36.790886 -3.100039	03/03/2023
El Toyo, Almería	AL4	36.847975, -2.332920	03/02/2023
<i>Asparagus aphyllus</i> (Prickly asparagus)			
Zahara de la Sierra, Cádiz	AP1	36.841757 -5.395525	20/03/2023
Mijas, Málaga	AP2	36.591142 -4.606242	23/03/2023
Torremolinos, Málaga	AP3	36.605964 -4.526782	20/03/2023
<i>Asparagus horridus</i> (Esparraguera)			
Cabo de Gata, Almería	H1	36.723495, -2.183220	19/03/2023
Vicar, Almería	H2	36.813587 -2.60462	11/03/2023
Enix, Almería	H3	36.875594, -2.609560	12/03/2023
Las Amoladeras Almería	H4	36.817729, -2.253485	07/03/2023
Rodalquilar, Níjar	H5	36.849231, -2.043093	148/02/2023
<i>Asparagus officinalis</i> (Garden asparagus)			
Láchar, Granada	O1	Purchased	01/04/2023
Loja, Granada	O2	Purchased	05/10/2023

#### 4.2. Extraction of Saponins

Sample preparation was carried out in triplicate to obtain the various *Asparagus* extracts. A weight of 0.5 g of wet *Asparagus* sample was solubilized in 20 mL of dichloromethane. The solution was extracted with an ultrasonic bath set at 50 °C for 5 min then filtered, the residue was again extracted with dichloromethane. Next, the residue was solubilized with 20 mL of methanol and extracted with an ultrasonic bath set at 50 °C for 5 min and again filtrated, the residue was again extracted with methanol. Filtrates were recovered and evaporated under reduced pressure with a rotary evaporator for dryness. Extract solutions were redissolved in methanol at 10 mg/mL. After centrifugation at 10.000 g for 5 min, supernatants were used for analysis (Le Bot et al., 2022).

#### 4.3. Total Saponin Content

TS of the *Asparagus* extracts was determined using a spectrophotometric method as described by Ncube et al. (2011) with minor modifications. Briefly, the dried methanolic extracts previously obtained were prepared at 10 mg/mL in methanol. Aliquots of 125  $\mu$ L were transferred to vials, followed by 125  $\mu$ L of freshly prepared vanillin in ethanol (0.8%, w/v) and 1.25 mL of sulphuric acid in water (72%, v/v). A control sample using methanol was also prepared. Samples were vortexed and heated at 60 °C for 10 min. Vials were cooled in ice for 5 min and absorbance was measured at 520 nm using a UV-VIS spectrophotometer against the control sample containing methanol. TS was obtained from a standard curve of diosgenin ranging from 100 to 1700  $\mu$ g/mL, which were prepared under the same conditions as previously stated for samples. Diosgenin was used as a representative standard of steroid saponins. Results were expressed as mg of total saponins per 100 g of dry sample. Determinations were done in triplicate.

#### 4.4. Characterization of Saponins by LC-MS

This methodology is fully detailed I Supplemental File 1. The chromatographic separations were performed on a Vanquish Flex Quaternary LC equipped with a reverse-phase C18 column (Hypersil Gold, 100 mm  $\times$  2.1 mm, 1.9  $\mu$ m) at a flow rate of 0.3 mL/min. The compounds were separated with gradient elution using acidified water (H<sub>2</sub>O containing 0.1% formic acid) (A) and acetonitrile (B) as eluents at room temperature (30 °C). The LC system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive<sup>TM</sup>, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion mode. Mass range in the full scan experiments was set at m/z 90–1000. LC chromatograms were acquired using the external calibration mode and they were processed using Xcalibur<sup>TM</sup> version 3.0, with Qualbrowser and Trace Finder 4.0 (Thermo Fisher Scientific, Les Ulis, France). Unknown analysis was carried out with Compound Discoverer<sup>TM</sup> version 2.1.

#### 4.5. Antitumor Assays

This methodology is fully detailed in Supplemental File 1. The antiproliferative activity of the saponin extracts from *Asparagus* shoots were assayed on the HT-29 human colon cancer cell line and the CCD-18 colonic human myofibroblasts cells line as described by Lyashenko et al. (2019).

#### 4.6. Statistical Analysis

Three aliquots for each sample were analyzed in triplicate for each location to get the results of saponins, and all data in the tables are reported as the mean value  $\pm$  SD. The significance of differences among mean values was assessed by one-way ANOVA coupled with Fisher's LSD test at  $P < 0.05$ . Pearson product-moment correlation ( $r$ ) and statistical significance ( $P$ ) were obtained for each pair of variables (the saponins).  $P < 0.05$  was regarded as significant. Cluster analysis was performed using Agglomerative Hierarchical Clustering AHC (Ward's technique) based on City-Block distance measure. All statistical analyses were carried out using Statgraphics<sup>®</sup> centurion XVI (StatPoint Technologies, Warrenton-Virginia, USA).

## 5. Conclusions

In this work, a total of 47 saponins were detected and quantified in the edible parts (shoots) of 4 taxa of wild *Asparagus* and cultured *A. officinalis*. The structure of all the saponins found contains skeletons of the furostane and spirostane type. The sum of dioscin and derivatives varied largely along the studied taxa, and these together with aspirostanosides (7 types detected) and aspirosides A, D, and M, constitutes the larger fractions of the *Asparagus* saponins detected in this work. Hierarchical cluster analysis of the saponin profiles clearly separated the various taxa and demonstrated that the taxonomic position is more important than the place from which the samples were acquired. Thus, the saponin profiles have chemotaxonomic significance in *Asparagus* taxa. The MTT assay showed dose- and time-dependent inhibitory effects of several saponins extracts on HT-29 cancer cells, standing out the cell growth inhibition exercised by *A. albus* and *A. acutifolius* after a 72-h period of cells exposure to extracts. Given the richness in saponins and antitumor activities, most *Asparagus* taxa analyzed here have potential used as functional foods. Further research involving purification of the various saponins fractions from several *Asparagus* extracts and one-to-one antitumor tests against several cancer cell lines could evidence more clearly their *in vitro* antiproliferative activity.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary File 1. Material and Methods, and Supplementary Tables.

**Author Contributions:** Conceptualization, R.L.R., I.R.G. and J.L.G.G.; methodology, T.C.C, R.L.R., I.R.G. and J.L.G.G.; software, T.C.C, R.L.R., A.M.G.C., I.R.G, M.A.R.C. and J.L.G.G. ; validation, A.M.G.C., I.R.G, M.A.R.C. and J.L.G.G.; formal analysis, T.C.C, R.L.R., A.M.G.C., I.R.G., M.E., and M.A.R.C.; investigation, J.L.G.G.; resources, R.L.R. and J.L.G.G.; data curation, T.C.C., M.A.R.C., A.M.G.C., M.E., and J.L.G.G.; writing—original draft preparation, I.R.G., A.M.G.C. and J.L.G.G.; writing—review and editing, J.L.G.G.; visualization, A.M.G.C., I.R.G., M.A.R.C., M.E., and J.L.G.G.; supervision, J.L.G.G.; project administration, J.L.G.G.; funding acquisition, R.L.R and J.L.G.G: All authors have read and agreed to the published version of the manuscript.

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