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NPC Natural Product Communications

2018
Vol. 13
No. 9
1105 - 1110

Biological Effects of Saponin Fractions from *Astragalus verrucosus* in Tumor and Non-tumor Human cells

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Received: May 1st, 2018; Accepted: July 23rd, 2018

Among natural chemicals used as cancer chemo-preventive and/or chemotherapeutic agents, saponins represent one of the most promising and interesting family of compounds. In this work, we aimed to elucidate the biological effects on human cells of six saponin fractions (SFs) obtained from *in vitro* cultures of *Astragalus verrucosus* Moris, a poorly characterized species. Interestingly, SF (**3**) showed a strongly inhibitory effect on the proliferation of human colon adenocarcinoma cell line (HCT116) via activation of a p53-dependent apoptotic pathway. In addition, SF (**3**) and the other SFs did not display genotoxic activity in human peripheral lymphocytes.

Keywords: Cytotoxicity, Genotoxicity, Apoptosis, Tumor cells, Human lymphocytes, Saponin fractions.

Natural compounds have been always considered a source of cancer chemotherapeutic and/or chemo-preventive agents. However, due to the increasing resistance of certain tumors and severe side-effects of conventional chemotherapy (including genotoxicity and secondary cancer formation), new pharmacological molecules are required. In the last decades, advances in cancer molecular biology have identified several compounds able to inhibit the growth of cancer cells with improved efficacy and selectivity, thus leading to less toxic forms of chemotherapy [1-6]. In this view, elucidation of the biological potential of newly natural occurring or synthetic chemical substances should be also devoted to ascertain the lack of cytotoxic/genotoxic effects, especially in non cancer cells. [7-9].

Secondary metabolites extracted from plants belonging to the genus *Astragalus* were proven to possess antioxidant, immunomodulatory, and antiviral properties, this making the herbs useful in the treatment of cardiovascular, neurodegenerative, infectious diseases and cancer. From a chemical point of view, the genus includes polysaccharides, saponins, and flavonoids. These components are considered the major chemical classes contributing to the multiple bioactivities of *Astragalus* species, although the respective molecular mechanisms of action are not fully elucidated [10]. Saponins are bioactive molecules, mainly of plant origin, but also produced by some marine organisms and insects. Other than cell membrane permeabilization, these compounds have shown cholesterol-binding, antiviral, immunomodulatory, antimicrobial, cytostatic, and antitumor activity [1,11-13]. Different studies reported the ability of saponins, in particular triterpenoid saponins, to inhibit the proliferation of several tumor cell lines through induction of cell cycle arrest and/or apoptosis [1,5,9,11-15]. Moreover, it has been shown that saponins can also have non-genotoxic and non-cytotoxic effects in human lymphocytes [16,17]. In addition to be used as single active molecule, some researchers have investigated the biological efficacy of saponins against cancer cells as either total extract or combinations of two or more different compounds [12,18-22]. In the present work, we aimed to elucidate the biological effects on human cells of some saponin fractions (SFs) obtained from *in vitro* cultures of *A. verrucosus*, a perennial herb located in a restricted area of Sardinia, which is a still poorly characterized species [23-25]. We therefore analyzed the tumor growth inhibitory activity of these SFs on a human colon adenocarcinoma cell line (HCT116) together with their genotoxicity in human peripheral lymphocytes.

Composition of SFs and biological activity in HCT116 cells: As shown in Table 1, SF (**1**), SF (**2**), SF (**3**), SF (**4**), SF (**5**) and SF (**6**) are composed by different combinations of known saponins extracted from *A. verrucosus* (Figure 1). Specifically, all SFs contain Astraverrucin IV (A IV), VI (A VI) and VII (A VII), Astrailienin A (AA) and Cicloaralosyde D (CD), whereas SF (**2**) and SF (**5**) also have Astraverrucin V (AV). The highest amount of saponin was found in SF (**6**): 897.4 µg of CD, while the lowest amount was found in SF (**5**): 42.8 µg of AVI.

Promotion of cell growth is a necessary feature of all types of cancer and can be attained by abnormally activated or deregulated signalling pathways involved in cell cycle regulation [14]. We therefore evaluated the antiproliferative effect of the six SFs from *A. verrucosus* on HCT116 cells using the sulforhodamine B (SRB) assay.

Table 1: Qualitative and quantitative composition of SFs.

SF	A IV		A V		A VI		A VII		AA		CD		Total	
	µg	%	µg	%	µg	%	µg	%	µg	%	µg	%	µg	%
1	179.8±7.4	22	0	0	83.5±3.3	10	157.3±6.9	19	155.3±6.9	19	237.1±7.8	29	813.0	100
2	470.1±20.2	25	174.3±7.1	9	79.3±3.1	4	224.7±9.9	12	390.2±13.7	21	560.1±18.4	30	1898.8	100
3	613.0±26.1	25	0	0	541.8±21.1	22	516.7±22.7	21	650.4±22.7	26	142.7±4.7	6	2464.6	100
4	512.1±22.0	23	0	0	123.4±4.8	6	219.0±9.6	10	541.4±18.9	25	810.3±26.7	37	2206.2	100
5	310.4±13.3	22	102.5±4.2	7	42.8±1.7	3	296.5±13.0	21	280.1±9.8	20	370.2±12.2	26	1402.5	100
6	471.5±20.3	19	0	0	321.7±12.5	13	305.4±13.4	12	513.1±23.0	20	897.4±34.1	36	2509.1	100

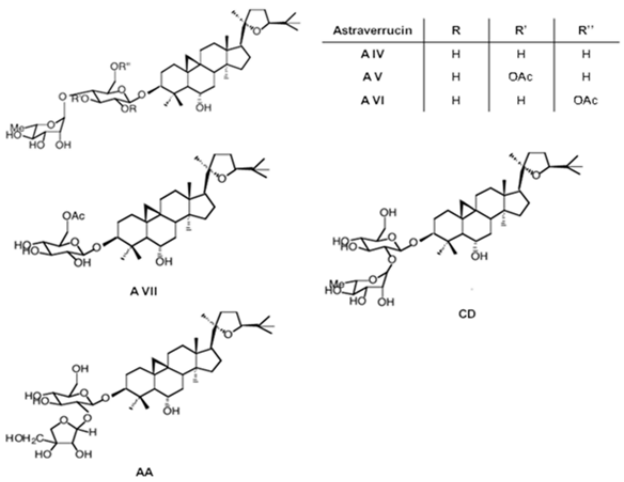

Figure 1: Structures of saponins extracted from *A. verrucosus*.

As shown in Figure 2, only SF (**3**) was able to significantly affect cell growth; the concentration that causes 50% growth inhibition (GI_{50}) was 7.92 ± 0.3 µg/mL for SF (**3**), whereas SF (**1**), SF (**2**), SF (**4**), SF (**5**) and SF (**6**) showed GI_{50} higher than 25 µg/mL.

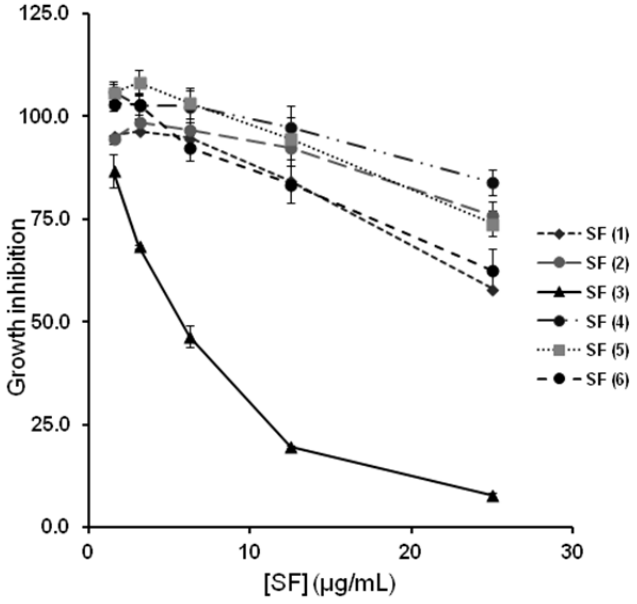

Figure 2: Growth inhibition curves, expressed as percent of the control (DMSO), in HCT116 cells treated for 48 h with different concentrations of the six SFs (see *Cell proliferation assay* of Experimental). The graph shows the data obtained treating cells with SFs doses up to 25 µg/mL. Values represent the mean ± S.E. of three independent experiments.

Doxorubicin was used as positive control and the relative GI_{50} was 0.054 µg/mL. As reported elsewhere [26], an extract that gave a GI_{50} of 30 µg/mL or less was considered active. Thus, we can conclude that SF (**3**) strongly inhibited the proliferation of HCT116 cells, especially when compared to the GI_{50} of other plant extracts presented in literature [20,21].

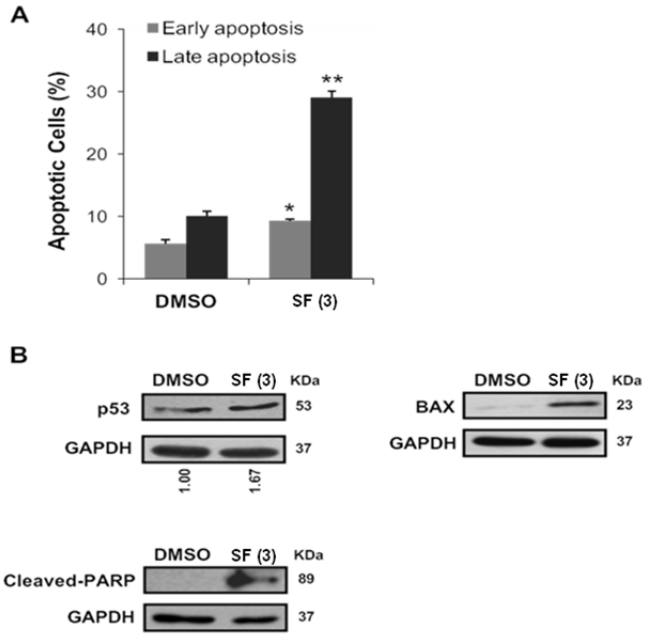

Figure 3: SF (**3**) induced early and late apoptosis in HCT116 cells. **(A)** Apoptosis was analyzed by flow cytometry using FITC-Annexin V and PI, after 48 h treatment with 15.84 µg/mL of SF (**3**) ($2xGI_{50}$) or DMSO only. Apoptotic cells are expressed as mean ± S.E.M. of two independent experiments; values are significantly different from DMSO (Student's t test; *$p < 0.05$; **$p < 0.01$). **(B)** SF (**3**) increased the expression levels of p53 and Bax, and led to PARP cleavage. Western blot analysis was performed after 24 h treatment with 15.84 µg/mL of SF (**3**) ($2xGI_{50}$) or DMSO only. The quantification of band intensity was performed using GAPDH as loading control.

Annexin-V/PI double staining demonstrated that SF(**3**) tumor growth inhibitory effect on HCT116 cells was associated to the induction of apoptotic cell death. In fact, as observed in Figure 3A, 15.84 µg/mL (twice the GI_{50}) of SF (**3**) induced early and late apoptosis. Western blot analysis showed that the SF (**3**)-induced apoptosis in HCT116 cells was associated with an increase in p53 protein, up-regulation of Bax (a p53 downstream pro-apoptotic target of the Bcl-2 family), as well as occurrence of PARP cleavage (a result of caspase-3 activation) (Figure 3B).

In addition, flow cytometric analysis showed that SF (**3**) did not interfere with the progression of HCT116 cells. As shown in Figure 4, in fact, the fraction of SF (**3**)-treated cells within each cell cycle stage was comparable with that of untreated cells (DMSO). All together, these findings support the notion that SF (**3**) is able to activate a p53-dependent apoptotic pathway.

Cytotoxicity/genotoxicity of SFs in human lymphocytes: Additionally, using the *in vitro* Cytokinesis-Block Micronucleus (CBMN) assay, we assessed the cytotoxic and genotoxic effects of the SFs in peripheral lymphocytes, a non-tumor and healthy human cell system. This assay is widely and routinely used in toxicology for the assessment of the genotoxic profile of chemical compounds

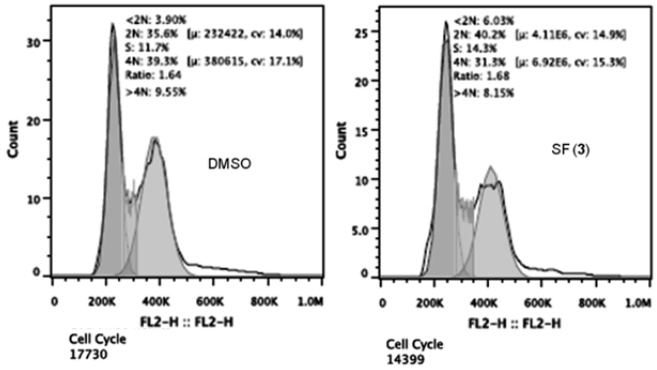

Figure 4: Representative DNA histograms of cell cycle analysis of HCT116 cells after a 48 h treatment with DMSO (control) or with the $2xGI_{50}$ concentration of SF (**3**). Cells were stained with propidium iodide and DNA content was then quantified by flow cytometry using the cell quest histogram analysis program.

of environmental or occupational concern [27]. The results of the CBMN test on SF-treated human lymphocytes are shown in Table 2. None of the selected SFs, tested up to a final concentration of 300 μg/mL, caused a significant increase in the frequencies of MN when compared to the respective untreated controls. On the other hand, MN frequency of MMC-treated cultures (positive control) was 14-20 times higher than the spontaneous MN frequencies. Interestingly, the window of cytostatic/cytotoxic effects was very narrow, as SFs started to induce appreciable cell cycle delay only at 150 μg/mL, while at 300 μg/mL we could not perform MN analysis due to a strong cell toxicity. Although the solubility of SFs in DMSO was a limiting factor, our results reasonably demonstrate that, under the present experimental conditions, the SFs 1) were not genotoxic for *in vitro* cultured human lymphocytes and 2) showed minimal or no cytostatic effect also at relatively high dosage. In agreement with these findings, either the total methanolic extract or the butanolic and ethyl-acetate subextracts from which SFs were isolated, assayed up to 900 or 600 μg/mL final concentration, respectively, did not cause increase in the spontaneous MN frequency (see Table 3).

Conclusions: In summary, five of the tested SFs (**1, 2, 4, 5,** and **6**) were not able to inhibit the proliferation of HCT116 cells. On the contrary, SF (**3**) exerts a strong anti-proliferative effect against this human cancer cell line inducing cell death via activation of a p53-dependent apoptotic pathway. Noteworthy, the anti-proliferative activity of SF (**3**) was associated to a lack of genotoxicity in *in vitro* cultured human lymphocytes. Comparing the SFs composition from a qualitative and quantitative point of view, it is interesting to observe that SF (**3**) is the extract containing the lowest quantity of CD together with a 1:1 ratio of AVI to AVII (Table 1). In this regard, SF (**1**) and SF (**5**) or SF (**6**), despite showing approximately the same percentage of AVII of SF (**3**) or a 1:1 ratio of AVI to AVII, respectively, have a high amount of CD. Furthermore, A IV, AA and AV, which are present (or not present) approximately in the same amount in all SFs, do not seem to interfere. Thus, we can hypothesize that is the concurrent presence of a low quantity of CD with a 1:1 ratio of AVI to AVII to play an active role in inhibiting the cell growth of HCT116 cells.

Table 2: Results of the CBMN test in human lymphocytes after treatment with SFs.

Dose (μg/mL)	SF (1)		SF (2)		SF (3)		SF (4)		SF (5)		SF (6)	
	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]
0.0[c]	4.25±1.11	2.21±0.07	4.25±1.11	2.21±0.07	6.00±0.91	2.08±0.08	6.00±0.91	2.08±0.08	4.50±1.19	2.19±0.12	4.50±1.19	2.19±0.12
0.6	6.25±1.03	2.16±0.10	5.50±1.19	2.03±0.09	7.75±1.31	2.19±0.11	5.00±1.47	2.20±0.02	4.75±0.85	2.16±0.10	4.25±1.03	2.19±0.11
3.0	6.75±1.31	2.07±0.01	4.50±0.87	2.01±0.12	5.75±1.38	2.18±0.12	4.75±1.18	2.01±0.13	6.50±1.44	2.17±0.13	7.25±1.49	2.27±0.04
15	6.00±1.15	2.14±0.09	5.50±1.12	2.13±0.13	5.75±1.93	2.09±0.04	7.50±1.76	2.20±0.02	7.00±1.47	2.18±0.04	6.00±0.71	2.26±0.01
75	4.50±0.87	2.07±0.20	4.75±1.77	1.99±0.06	7.25±1.80	1.91±0.02	6.50±1.55	1.76±0.04	5.00±1.83	2.05±0.20	7.50±1.44	2.19±0.08
150	7.00±1.08	1.63±0.20	6.50±1.19	1.67±0.19	8.25±1.65	1.52±0.06	7.25±1.38	1.58±0.08	7.25±1.65	1.73±0.17	6.25±0.85	1.68±0.07
300	n.a.[e]		n.a.[e]		n.a.[e]		n.a.[e]		n.a.[e]		n.a.[e]	
MMC (0.2)[d]	87.5±11.4[f]	1.55±0.13	87.5±11.4[f]	1.55±0.13	76.5±8.8[f]	1.44±0.10	76.5±8.8[f]	1.44±0.10	93.0±12.1[f]	1.51±0.05	93.0±12.1[f]	1.51±0.05

[a]Micronuclei frequency (MN): average number of micronucleated binucleated cells per 1000 binucleated scored (two independent experiments).
[b]Cell proliferation Index (CBPI): values show cytostatic/cytotoxic effect in the cells.
[c]Negative control: DMSO 1%.
[d]MMC: mitomycin C (positive control).
[e]n.a.: not analyzable.
[f]Significantly different from the negative control ($p< 0.001$).

Table 3: Results of the CBMN test in human lymphocytes after treatment with the methanol, ethyl- acetate and butanol extracts.

Dose (μg/mL)	Methanol		Ethyl- acetate		Butanol	
	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]	MN (‰)[a]	CBPI[b]
0.0[c]	4.25±0.48	2.26±0.11	4.50±1.04	2.19±0.07	3.50±0.64	2.26±0.11
0.6	3.50±1.04	2.27±0.01	0.5±0.5	2.22±0.06	5.25±1.11	2.25±0.14
3	6.75±1.31	2.22±0.04	3.0±0.0	2.06±0.03	4.00±0.91	2.17±0.09
15	4.50±0.87	2.14±0.03	1.5±0.5	2.20±0.03	3.75±1.03	1.91±0.08
75	7.25±1.31	2.13±0.03	2.0±1.0	1.98±0.08	6.00±1.22	1.85±0.16
300	5.00±1.08	1.92±0.14	4.0±0.0	1.66±0.12	4.50±1.76	1.44±0.09
600	5.25±1.25	1.56±0.04	2.0±2.0	1.48±0.03	4.00±1.58	1.31±0.06
900	4.00±1.08	1.37±0.12	toxic		toxic	
MMC (0.2)[d]	81.5±8.2[e]	1.42±0.08[e]	98.0±13.0[e]	1.51±0.14[e]	79.5±10.5[e]	1.39±0.1[e]

[a]Micronuclei frequency (MN): average number of micronucleated binucleated cells per 1000 binucleated scored (two independent experiments).
[b]Cell proliferation Index (CBPI): values show cytostatic/cytotoxic effects in the cells.
[c]Negative control: DMSO 1%.
[d]MMC: mitomycin C (positive control).
[e]Significantly different from the negative control ($p< 0.001$).

This study is the first to investigate biological activities of SFs obtained from *in vitro* cultures of *A*. *verrucosus* and may pave the way for further investigations on extracts containing secondary metabolites belonging to a single chemical class. For example, it might be interesting to test directly the two (putative) active ingredients of SF (**3**) on HCT116 and other cancer cell lines, either alone or in combination with known anticancer drugs.

Experimental

Plant material: *Astragalus verrucosus* Moris harvested in Italy (Is Pisittus, Sardinia Island) was collected in June 1994 and a voucher specimen is deposited in the Herbarium Of Istituto di Botanica ed Orto Botanico, Università di Urbino, Italy (Register n° URB-96/357).Cotyledons from germinated in vitro seedlings-plants and micropropagated leaves from Sardinian wild *Astragalus verrucosus* Moris seeds have been transformed by co-cultivation with three wild type *Agrobacterium rhizogenes* strains (ATCC 11325, ATCC 15834 and NCPP 1855). Hairy roots have been induced by each strain and were cloned and multiplied either in solid and in liquid medium following reported procedures [28].

General experimental procedures: The saponin standards [astraverrucins (A IV-VII), cycloaralosyde (CD), astrailienin (AA)] were isolated, identified and purified in our laboratory from the aerial parts of wild *A*. *verrucosus* plants [23,24]. The TLC screening was performed by Kieselgel 60 Si F254 and RP-18 F254 (Merck) plates eluted with a range of mixtures. The plates were visualized under UV light (254 and 366 nm) and were detected by specific spray reagents (Ceric sulphate, Komarowsky Reagent, Naturstoff reagents-PEG). Gel permeation was performed by Sephadex LH-20 (10 mm x 300 mm, 25-100 mm, Pharmacia, Fine Chemicals) at 1.0 mL/min flow. All reagents used were of analytical-HPLC reagent grade. LC-DAD-MS experiments were carried out by a Surveyor Pump and Autosampler Thermofinnigan equipped with PDA detector as well as LCQ Advantage Thermofinnigan mass detector (ESI interface). 300 mm, 25-100 mm, Pharmacia, Fine Chemicals) at 1.0 mL/min flow. All reagents used were of analytical-HPLC reagent grade.

Extraction and chemical analysis: Three different strain of *A*. *rhizogenes* to produce hairy roots were tested by the cotyledons and leaf explants of *A*. *verrucosus*. Six clones of *A*. *verrucosus* hairy roots (AVHR) were lyophilized and then extracted in a Soxhlet apparatus with *n*-hexane, and methanol in turn. After removal of solvent *in vacuum* at temperatures up to 40°C, the methanol residue was partitioned with H_2O saturated EtOAc and then with H_2O saturated *n*-BuOH.

These residues were purified by gel filtration over Sephadex LH-20 (Pharmacia, Fine Chemicals, 30 cm x 1 cm, flow 1.0 mL min-1) eluted with methanol and with MeOH-W (4:1) respectively. In order to select purified mixtures of saponin compounds, the composition of the different eluted fractions were compared by both Kieselgel 60 Si [CHCl3-Et2O (4:1)] and RP-18 TLC plates [MeOH-W (4:1)] visualized under UV light (254 and 366 nm) and detected by Naturstoff reagents-PEG and Komarowsky spray, using reference compounds mentioned above. The selected fractions were diluted in MeOH HPLC grade and analyzed by LC-ESI-MS using five - level external calibration curves with standard solution ranging from 62 to 112 µg/mL of each saponins. A gradient LC elution program was used by CH3CN-W- HCOOH 0.1% mixtures (from 30:70 to 60:40) for 60 min. Injection volume 20 µL. The MS tuning section was performed by the direct injection of a standard solution of AV (sheath gas flow 20 arb, capillary temperature 280°C, negative mode).

Human tumor cells and growth conditions: The p53 proficient human colon adenocarcinoma cell line, HCT116, was kindly provided by Dr. B. Vogelstein (The Johns Hopkins Kimmel Cancer Center, Baltimore, Maryland, USA) and was routinely cultured in RPMI-1640 with ultra-glutamine medium (Lonza, VWR, Carnaxide, Portugal) supplemented with 10% foetal bovine serum (FBS) (Gibco, Alfagene, Carcavelos, Portugal) and maintained in a humidified incubator at 37 °C with 5% CO_2 in air.

Cell proliferation assay: We performed the SRB assay as described elsewhere [28]. Briefly, HCT116 cells were plated in 96-well plates at a final density of 5.0×10^3 cells/well. After 24 h, the cells were treated with serial dilutions of SFs (ranging from 0 to 100 µg/mL) or Doxorubicin (0-300 nM) as positive control for 48 h. The solvent of the tested chemicals (DMSO) corresponding to the maximum concentration used in the assay (0.5%) was included as negative control. The concentration of compound that causes a 50% reduction in the net protein increase within the cells (GI_{50}) was determined.

Flow cytometric analysis: HCT116 cells were seeded in 6-well plates at a final density of 1.5×10^5 cells/well and incubated for 24 h. Cells were then treated with 15.84 µg/mL, twice the GI_{50} concentration ($2xGI_{50}$), of SF (3) or DMSO only (control) for 48 h. For apoptosis analysis, cells were analyzed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Enzifarma, Porto, Portugal), according to the manufacturer's instructions. As described elsewhere [29], the distribution of apoptotic cells was identified by flow cytometer on FACSCalibur (BD Biosciences, Enzifarma, Porto, Portugal) and analyzed with the Cell Quest software (BD Biosciences, Enzifarma, Porto, Portugal). For cell cycle analysis, cells were thereafter fixed in ice-cold 70% ethanol and incubated at 37 ºC with RNase A (Sigma-Aldrich, Sintra, Portugal) at a final concentration of 20 µg/mL for 15 min, and further incubated with 50 µg/mL of propidium iodide (PI) (Sigma-Aldrich, Sintra, Portugal) for 30 min, followed by flow cytometric analysis.

Western Blot analysis: To analyze the protein expression in HCT116 human tumor cell lines, 1.5×10^5 cells/well were plated in 6-well plates, allowed to adhere for 24 h, and then incubated with 15.84 µg/mL of SF (**3**) or DMSO only (control) for 24 h. Whole cell lysates were prepared as described elsewhere [28]. Following whole protein quantification using the Coomassie staining (Bradford, Sigma–Aldrich, Sintra, Portugal), proteins (40 µg) were electrophoresed on 10% SDS–PAGE and transferred to a Whatman nitrocellulose membrane (Protan, VWR, Carnaxide, Portugal). Membranes were blocked with 5% milk and probed with a mouse monoclonal anti-p53, anti-Bax), or anti-PARP antibodies (Santa Cruz Biotechnology, Frilabo, Porto, Portugal), followed by an anti-mouse horseradish-peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Frilabo, Porto, Portugal). For loading control, membranes were stripped and re-probed with a mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Frilabo, Porto, Portugal). The signal was detected with the ECL Amersham kit (GE Healthcare, VWR, Carnaxide, Portugal) and the Kodak GBX developer and fixer (Sigma–Aldrich, Sintra, Portugal). Band intensities were quantified using the ImageJ software.

Human lymphocytes cultures and chemical treatments: Heparinized peripheral blood samples were obtained from healthy young non-smoking male donors. The protocol was approved by the Azienda Ospedaliero-Universitaria Pisana ethical committee. After adding 0.3 mL of whole blood to 4.7 mL of RPMI 1640 (Invitrogen,

Milano, Italy) supplemented with 20% foetal bovine serum (Invitrogen, Milano, Italy), 1.5% phytohaemagglutinin (PHA) (Invitrogen, Milano, Italy) and 1.0% penicillin/streptomycin (Invitrogen, Milano, Italy), stimulated lymphocytes were incubated and cultured at 37°C for 72 h. After performing a cytotoxicity screening, SFs were dissolved in DMSO up to 300 µg/mL final concentration and added to lymphocyte cultures after 24 h. An approximately 20-30% reduction of cell proliferation was obtained for all the SFs at 150 µg/mL. Mitomycin C (MMC) was used as positive control at 0.2 µg/mL. Control cultures not receiving chemicals were also set up according to the maximum concentration of solvent used (DMSO, not exceeding 1% final concentration).

Cytogenetic assay: Cytochalasin B (6 µg/mL; Sigma Aldrich, Milano, Italy) was added at 44 h to all culture tubes to block cell cytokinesis. As described in details elsewhere [30], lymphocytes were harvested at the end of cell culturing by 4 min centrifugation at 2400 rpm, treated with 10 mL of 0.075 mM KC1 for a few min to lyse erythrocytes, prefixed in methanol/acetic acid 3:5, fixed in 100% methanol, washed twice in methanol/acetic acid 7:1, and dropped onto clean glass slides. The air-dried slides were then stained in 5% Giemsa.

Slide scoring: For each experimental point, 2000 binucleated cells (1000 cells from two replicate cultures) were scored for the presence of MN according to standard criteria [31]. The cell proliferation index (CBPI) was calculated as follows: (M + 2B + 3P) / (M + B + P), where M, B and P are the number of cells that have not yet entered the first mitosis, (M, mononucleated), and cells that have divided once (B, binucleated) and twice (P, plurinucleated, these latter comprising both tri- and tetranucleated), respectively. (M + B + P) represents a total of at least 1000 cells scored. MN frequency was express as number of micronucleated binucleated cells per 1000 binucleated cells scored.

Statistical analysis: Statistical elaborations were performed by the STATGRAPHICS Plus version 5.1 software package (Statistical Graphics Corporation, 2001, Rockville, USA). The Student's t-test was used to analyze the antiproliferative activity of SFs on HCT116 cells. Genotoxicity data were elaborated by the Dunnett test, which performs a multiple comparative calculation of the MN or CBPI values from treated *versus* control cultures. *P* values less than 0.05 were considered as statistical significant. Data were reported as mean ± S.E. of three (tumor cell lines) or two (human lymphocytes) independent experiments.

Abbreviations: Bax, Bcl-2 Associated X protein; Bcl-2, B-Cell CLL/Lymphoma 2; DMSO, Dimethyl Sulfoxide; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; PARP, Poly ADP (Adenosine Diphosphate) - Ribose Polymerase; PBS, Phosphate-Buffered Saline; PI, Propidium Iodide; TLC, Thin Layer Chromatography.

Acknowledgments - This work received the financial support from the European Union (FEDER funds through the COMPETE program) and National Funds (FCT, Fundação para a Ciência e Tecnologia) through REQUIMTE (Pest-C/EQB/LA0006/2013). It was also supported by FCT fellowships to J. Soares (SFRH/BD/78971/2011) and to S. Gomes (SFRH/BD/96189/2013).

Conflict of interest: The authors declare that there is no conflict of interest.

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