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In Vitro Cytotoxic Effects of Secondary Metabolites Present in *Sarcopoterium Spinosum* L.

Jozef Hudec ¹, Jan Mojzis ² , Marta Habanova ^{3,*} , Jorge A. Saraiva ⁴ , Pavel Hradil ⁵, Tibor Liptaj ⁶, Lubomir Kobida ⁷ , Miroslav Haban ^{7,8}, Maria Holovicova ⁹ and Dominika Zvercova ⁷

- ¹ Department of Agrochemistry and Plant Nutrition, Faculty of Agrobiolgy and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; jahc.13@gmail.com
- ² Department of Pharmacology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Trieda SNP 1, 040 11 Košice, Slovakia; jan.mojzis@upjs.sk
- ³ Department of Human Nutrition, Faculty of Agrobiolgy and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia
- ⁴ QOPNA & LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal; jorgesaraiva@ua.pt
- ⁵ Department of Organic Chemistry, Faculty of Science, Palacky University Olomouc, Tr. 17. Listopadu, 1192/12, 771 46 Olomouc, Czech Republic; pavel.hradil@upol.cz
- ⁶ Central Laboratories, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 812 37 Bratislava, Slovakia; tiber.liptaj@stuba.sk
- ⁷ Department of Sustainable Agriculture and Herbology, Faculty of Agrobiolgy and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; lubomir.kobida@uniag.sk (L.K.); miroslav.haban@gmail.com (M.H.); xzvercova@uniag.sk (D.Z.)
- ⁸ Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University in Bratislava, 832 32 Bratislava, Slovakia
- ⁹ AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; mariaholovicova23@gmail.com
- * Correspondence: marta.habanova@uniag.sk; Tel.: +421-376414467

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Abstract: *Sarcopoterium spinosum* (L.) is a medicinal plant traditionally used for the treatment of various diseases including cancer in the Near- and Middle East. The fractions and constituents of the ethanol extract of *S. spinosum* were screened for in vitro cytotoxic activities on Jurkat (acute T-lymphoblastic leukemia), HeLa (cervical adenocarcinoma), MCF-7 (mammary gland adenocarcinoma), Caco-2 (human colorectal adenocarcinoma), and MDA-MB-231 (mammary gland adenocarcinoma) cell lines using the MTT (3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The ethanol extract was subsequently re-extracted with ethyl acetate and in its sub-fraction obtained by column chromatography three compounds (stachydrine, benzalkonium chloride and rutin) were the first time identified by nuclear magnetic resonance (NMR) analyses. The most active subfraction showed cytotoxic activity against HeLa, MCF-7, and Caco-2 cell lines. The three compounds mentioned, as standards of high-performance liquid chromatography (HPLC) quality, were studied individually and in combination. Cytotoxic activity observed might be due to the presence of benzalkonium chloride and rutin. Benzalkonium chloride showed the strongest growth suppression effect against HeLa cells (IC₅₀ 8.10^{−7} M) and MCF-7 cells (IC₅₀ 5.10^{−6} M). The mixture of stachydrine and benzalkonium chloride allowed a synergistic cytotoxic effect against all tested cancer and normal cells to be obtained. Anti-cancer activity of the plant extract of *S. spinosum* remains under-investigated, so this research describes how the three major compounds identified in the ethyl acetate extract can exert a significant dose dependent in vitro cytotoxicity.

Keywords: *Sarcopoterium spinosum* (L.); medicinal plants; cytotoxicity; inhibition; stachydrine; benzalkonium chloride; rutin; cancer cells

1. Introduction

Cancer is the second leading cause of death [1], accounting for nearly 10 million deaths in 2020 worldwide [2], despite advances in chemotherapy development and tumor biology research. Surgery, radiotherapy, and chemotherapy are still the main forms of treatment. New discoveries in signalling pathways involved in tumor development, proliferation, and metastasis and also the completion of the Human Genome Project partially ameliorated some therapeutic problems. Nevertheless, the fundamental problems remain associated with the heterogeneity of the tumor, drug resistance, and systemic toxicities, since it often contains a diverse mixture of cells, and only a part of them can bounce back after treatment. The multiple, interconnected pathways presented in these cells complicate the efforts for providing effective therapies [3]. Troubleshooting remains based on the development of such small molecules which inhibit selectively and the most complexly undesired biological processes in a tumor cell, being able to selectively kill only tumor cells but not their healthy counterparts [4].

The cytotoxic-active compounds isolated from non-edible plant parts can also be used as health benefit agents, such plants as *Sarcopoterium spinosum* (L.). This plant (syn: *Poterium spinosum* L.) is a spiny rosaceous dwarf shrub, and it is thought to have originated from the semi-arid transition zone between the Mediterranean and Irano-Turanian steppe vegetation zone, and then expanded westward [5]. Chemical constituents of *S. spinosum* and their bioactivity have been described in several works. Five pentacyclic triterpenoids [6], such as tormentic and urosolic acid, further beta-sitosterol [7], catechins, epicatechins [8], and proanthocyanidins [9] and other substances were identified in the root and aerial part of the extract. Most of the bioactive components are located in the thorns and leaves. Aldal'in [10] reported a gas chromatography–mass spectrometry (GC-MS) chromatogram of thorns and leaves methanolic extract of *S. spinosum* and showed seven major peaks for thorns and six major peaks for leaves, while three of major constituents (thymol, boric acid and dodecenedioic acid) were found in the extract of both the parts. The compounds of β -D-glucopyranoside, linoleic acid and cinnamic acid were found as major compounds only in thorns extract. Otherwise, benzoic acid, palmitic acid and malonic acid were found only in leaf extracts as major phytoconstituents. Henkin et al. [11] reported that tormentic acid from *S. spinosum* is suggested to be the major constituent extract, and to mediate its anti-proliferative activity on several cancer cell lines. Tormentic acid plays an important role in the cytotoxicity exhibited by the extract [12]. *S. spinosum* extracts have been used in medical practice for the treatment of several disorders. Among other applications, the therapeutic applications in ethnopharmacological surveys are mostly for diabetes [13,14] and disorders of the digestive system [15], pain relief [16], poisoning [17], anti-inflammatory effects [18], for the prevention and treatment of hepatic steatosis [19], and cancer [12]. Moreover, the anti-cancer effect of *S. spinosum* extract has also been reported. Durodola showed significant tumor inhibitory activity of a crude extract of *S. spinosum* in experimental animals [20].

The purpose of the present study was to investigate aerial parts of *S. spinosum*, perform an in vitro cell proliferation assay testing all extracted fractions and subfractions, perform the identification and subsequent synthesis of the molecule as a possible way to obtain the basic material for the synthesis of new anti-cancer compounds, and study three identified compounds in the most cytotoxic subfraction individually and in combination.

2. Materials and Methods

2.1. Plant Material

The aerial parts of *S. spinosum* were collected from the basis of Mount Tavor (Shibli–Umm al-Ghanam, Israel) (32°41'37.375" N and 35°24'41.5645" E) during fruiting periods in June 2012, and taxonomically identified by Professor Dr. T. Baranec, Slovak University of Agriculture in Nitra, Slovakia. A voucher specimen (DB-09-07-2012) of the plant is deposited at the Department of Botany, Slovak Agricultural University in Nitra. The plants were dried at room temperature, powdered, and homogenised in a grinder IKA A10 basic (IKA, Staufen, Germany).

2.2. Extraction and Isolation of Bioactive Compounds

The bioactive compounds were extracted and fractionated according to their polarity as shown in Figure 1. Briefly, the powdered aerial parts including leaves and thorns of *S. spinosum* (1.6 kg) were sonicated with petroleum ether (40–65 °C) (3 × 1.5 L, 1 h each). The resulting supernatants were collected and filtered through absorbent cotton, followed by evaporation of the solvent at 35 °C under low pressure. The resulting liquid residue was labelled as the PF (petroleum ether fraction, 15 g). The defatted material remaining from petroleum ether extraction was re-extracted with 70% ethanol (3 × 2 L, 3 h under reflux each). This 70% ethanol extract was then filtered through absorbent gauze, and the filtrate was concentrated under low pressure to remove ethanol.

Figure 1. Extraction, fractionation, and column chromatography separation of the aerial parts extracts of *S. spinosum* (L.).

The resulting suspension was successively partitioned in 3 sequentially partitions with ethyl acetate and 1-butanol and the respective extracts combined. The ethyl acetate and 1-butanol extracts were evaporated to dryness under low pressure, while the aqueous layer was lyophilized. These 3 fractions were designated as AF (ethyl acetate fraction) (60 g), BF (butanol fraction) (92 g) and WF (water fraction) (268 g), respectively.

Since the highest cytotoxic activity was found in the AF, this fraction was subjected to column chromatography (CC), repeated many times due to the larger amount of extract, using silica gel (75–150 µm, 2 × 50 cm, flow rate 0.7 mL/min), the dose of solid AF was 0.5–0.7 g/column, firstly eluted with increasing polarities of a mixture of petroleum ether and ethyl acetate resulting in 13 fractions, and subsequently with increasing polarities of a mixture of ethyl acetate and methanol resulting in eight fractions (Fr.14–Fr.21) and followed with a mixture of acetone and water (1:1), resulting in two fractions (Fr.2–Fr.23). The most cytostatic-active fraction, the Fr.18 (ethyl acetate/methanol (5:5), 45 mL/0.2 g of sample/separation cycle) was evaporated to dryness under low pressure. Then, the Fr.18 was separated by silica gel CCa (the same parameters as above) and eluted with a mixture of chloroform/methanol (10:1), with two fractions being collected to be studied further (Fr.18a that was designated as M8R and Fr.18b designated as M8N).

2.3. Tumor Cell Lines

Jurkat (acute T-lymphoblastic leukemia), HeLa (cervical adenocarcinoma), cells were maintained in RPMI 1640 medium supplemented with Glutamax-I. Caco-2 (human colorec-

tal adenocarcinoma), MCF-7 (mammary gland adenocarcinoma), MDA-MB-231 (mammary gland adenocarcinoma), were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I and glucose. Both media were supplemented with 10% (*v/v*) fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Invitrogen, Carlsbad, California, UK), in humidified air with 5% CO₂ at 37 °C. Before each assay, cell viability was determined by the trypan blue (Invitrogen, UK) exclusion method and found to be greater than 95%. 3T3 (murine fibroblasts) cell line was maintained in a growth medium consisting of high-glucose DMEM with sodium pyruvate (GE Healthcare, Piscataway, NJ, USA). The growth medium was supplemented with a 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Invitrogen, Carlsbad, CA, USA) in an atmosphere containing 5% CO₂ in humidified air at 37 °C. Cell viability, estimated by trypan exclusion, was greater than 95% before each experiment.

2.4. Cell Proliferation Assay

Studied compounds were dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.02% and exhibited no cytotoxicity. The effect of each fraction or individual compounds on cell proliferation was determined using a colorimetric microculture assay with the MTT (3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) endpoint [21]. Briefly, 5×10^3 cells were plated per well in 96-well polystyrene microplates (Sarstedt AG & Co., Nümbrecht, Germany). Twenty-four hours after seeding, different doses (0.0019 to 1.00 mg/mL) of the tested chemicals were added. After 72 h of incubation, 10 µL of MTT (5 mg/mL; Sigma Aldrich Chemie, Steinheim, Germany) were added to each well. After an additional 4 h period, during which insoluble formazan was produced, 100 µL of 10% sodium dodecylsulphate were added to each well and another 12 h of incubation enabled the formazan to dissolve. The absorbance was measured at 540 nm using the automated µQuant™ Universal Microplate Spectrophotometer (Biotek, Oxfordshire, UK). The calculation formula is: cell proliferation rate = (experimental group A_{540 nm}-zero adjustment group A_{540 nm})/(Control group A_{540 nm}-zero adjustment group A_{540 nm}) × 100%. Absorbance of control wells was taken as 100%, and the results were expressed as percentage of control.

2.5. Synthesis of Stachydrine Hydroiodide

Synthesis was carried out following the procedure described by [22] with modifications in the purification process. Briefly, L-proline (1 g; 8.69 mM) was dissolved in a solution consisting of sodium hydroxide (1.03 g; 25.75 mM) and dry methanol (10 mL). Methyl iodide (1.60 mL; 25.59 mM) was added at room temperature and the mixture was stirred for 6 h under gentle reflux. After cooling to room temperature, more methyl iodide (0.54 mL; 8.64 mM) was added and the mixture was again stirred for 6 h under gentle reflux. The reaction mixture was concentrated to dryness using a rotary evaporator (RVO 400, INGOS, Czech Republic). The light-yellow solid residue was dissolved in methanol (15 mL) and after addition of ethyl ether a white solid was precipitated, filtered and then washed with ethyl ether (2 × 5 mL). The dry crude product was suspended in acetone (50 mL), boiled (5–10 min) and filtered while hot. The filter cake was washed with acetone (2 × 15 mL) and allowed to dry in the air. The yield obtained was 1.92 g (i.e., 82%) of the white crystals with a sharp melting point (224 °C).

2.6. Nuclear Magnetic Resonance (NMR) Analysis

¹H and ¹³C spectra of sub-fraction M8R were obtained using a Varian VNMRs 600 instrument at the Faculty of Chemical and Food Technology STU in Bratislava. According to the detected nucleus, "Triple-resonance HNC" probe (¹H and "indirect detected" heteronuclear experiments) or "Double resonance DBC" probe (¹³C detected experiments) was used. Besides ¹H and ¹³C NMR experiments, additional experiments: gCOSY, gDQF-COSY, 2D TOCSY, HSQC and HMBC were performed if required (copies of the original spectra can be obtained from the corresponding author). All NMR experiments were performed by pulse

programs provided by the spectrometer manufacturer. The standard operating parameters were used, and all NMR measurements were performed at 25 °C. Samples dissolved in different deuterated solvents (deuterated methanol (Met-d₄), deuterated chloroform (CDCl₃), deuterated dimethyl sulfoxide (DMSO-d₆), and deuterated water (D₂O)) were studied. NMR spectra of the major compound of sub-fraction M8R, proline betaine, synthesized as iodide at the Faculty of Science, Palacky University in Olomouc were obtained using a JEOL 400 MHz instrument (JEOL, Tokyo, Japan). Measurements were undertaken at room temperature in a D₂O solution. Unambiguous signals assignments were achieved with the help of 2D heteronuclear (¹H-, ¹³C-) and (¹H-, ¹⁵N-) HMBC experiments.

2.7. Ultra High-Performance Liquid Chromatography–Mass Spectrometry (UPLC-MS) Analysis

Analysis of stachydrine hydroiodide was also performed using a Waters Acquity UPLC-PDA-QDA system (Waters Corporation, Milford, MA, USA). The system used electrospray ion source (ESI) in positive and negative modes. Analyses were performed for the following conditions: security cartridge C18-4 × 3 mm (Phenomenex) and X Select HSS T3 column (2.5 µm, 3 × 50 mm, 30 °C). Mobile phase: linear gradient from 20 to 80% of acetonitrile in 0.01 M ammonium acetate, and a flow rate 600 µL/min was used. MS parameters: TESI 600 °C, Tcap 450 °C, (±) ESI ± 3.0 kV. For the analysis was used solution of stachydrine hydroiodide 0.5 mg in 1 mL of the solvent (20% acetonitrile and 80% water). Time of the analysis 5.5 min. Major component: UPLC-(+ESI)-MS: m/z 144 (M+H⁺); 166 (M+Na⁺); 287 (2M+H⁺); 309 (2M+Na⁺); UPLC-(-ESI)-MS: m/z 126.8; 140.8; 276.7. The positive mode was used for the confirmation of stachydrine, and the negative mode was used for the confirmation of the presence of hydrogen iodide.

2.8. Chemical Materials

MTT (purity > 97.5%), alkyl-benzyl-dimethyl-ammonium chloride (benzalkonium chloride) (purity > 95%), and Paclitaxel (purity > 97%) were purchased from Sigma-Aldrich (Steinheim, Germany); and rutin sulphate sodium salt from Merck (Darmstadt, Germany). L-proline (purity > 99%) and methyl iodide (purity > 99%), purchased from Sigma-Aldrich, St. Louis, MO, USA, were used to prepare L-proline betaine iodide (stachydrine hydroiodide or N, N-dimethyl-L-proline hydroiodide). All other solvents, petroleum ether 40–65 °C, ethyl acetate, ethanol, methanol, acetone and 1-butanol (Chemical Laboratories, Adamík, Slovakia) were of analytical reagent grade and used without any further purification.

2.9. Statistical Analysis

All experiments were performed in triplicate and the values of cell growth inhibition are expressed as mean ± standard deviation (SD). The IC₅₀ values were calculated using Graphpad Prism 5.04 (San Diego, CA, USA) software. Differences among means were tested for statistical significance (*p* < 0.01) using one-way analysis of variance (ANOVA).

3. Results

3.1. Isolation of Cytostatic Compounds and Cell Proliferation Analysis

The aerial parts of the plant material including leaves and thorns of *S. spinosum* were air-dried, ground to a powder and firstly extracted with petroleum ether. The defatted leftover was then re-extracted with 70% aqueous ethanol. The ethanol extract was further fractionated by being partitioned with ethyl acetate and 1-butanol, and all the obtained fractions PF (petroleum ether fraction), AF (ethyl acetate fraction), BF (1-butanol fraction), and WF (water fraction) were evaluated for their cytotoxic activity by MTT and spectrophotometric assays.

The average IC₅₀ values for MCF-7 were found to be 22.0 ± 6.5, while for both HeLa and Caco-2 cell lines the IC₅₀ was above 1000 µg/mL (Table 1).

Table 1. IC₅₀ value (µg/mL) of the ethyl acetate fraction (AF) of *S. spinosum* L. extract and the more active fractions obtained from the column chromatography separation in various cancer and non-cancer cell lines.

Sample	IC ₅₀ Value ^a (µg/mL)				
	HeLa ^b	MCF-7 ^c	Caco-2 ^d	MDA ^e	3T3 ^f
M-AF	>100	22.0 ± 2.7	>100	Nt	nt
M8	20.7 ± 2.5 *	5.9 ± 1.1 *	56.0 ± 4.2 *	Nt	nt
M10	>100	21.2 ± 2.4	>100	Nt	nt
M8R	5.8 ± 1.1 *	2.2 ± 0.8 *	9.5 ± 1.4 *	62.5 ± 3.8	270.0
M8N	>100	>100	>100	>100	>500

^a Data are the mean ± SD (n = 3). ^b Cervical carcinoma cells. ^c Breast cancer cells. ^d Human colorectal adenocarcinoma. ^e Mammary gland adenocarcinoma. ^f Mouse fibroblast cells. * Data with statistical significance against control (crude extract, M-AF): *p* < 0.01. nt-not tested. Fr. 18 was designed as M8; Fr. 20 was designed as M10.

In the present study, further experiments were carried out on the AF extract in order to separate its cytotoxic components, being a column chromatography performed, resulting in the isolation of 23 sub-fractions that were studied concerning its effects on cell proliferation. Two fractions (Fr.18 and Fr.20) showed higher potential of cytotoxic activity than the others, Fr. 18 in all tested cells. The most cytotoxic-active fraction (Fr.18, respectively M8) showed the average IC₅₀ values against HeLa, MCF-7, and Caco-2 cell lines as followed: 20.7 ± 2.5, 5.9 ± 1.1 and 56.0 ± 4.2 µg/mL, respectively. This fraction was subsequently purified and the first sub-fraction, designated as M8R, showed IC₅₀ values against HeLa, MCF-7, Caco-2, and MDA cell lines as followed: 5.8 ± 1.1, 2.2 ± 0.8, 9.5 ± 1.4, and 62.5 ± 3.8 µg/mL, respectively (Table 1).

3.2. Compounds Present in the Isolated Fraction

Compounds present in the M8R sub-fraction were identified by spectroscopic analyses. From series of NMR spectra (standard ¹H and ¹³C, 2-dimensional COSY, HSQC and HMBC) it was possible to obtain a general idea of M8R composition. The major components found in M8R were stachydrine, benzalkonium chloride, and rutin (Figure 2).

Figure 2. ¹H NMR (nuclear magnetic resonance) spectra of the most cytotoxic sub-fraction (M8R) in CD₃OD, isolated from *S. spinosum* after fractionation and purification. Alkane chain is correlated with aliphatic chain of benzalkonium chain. Phthalate signals—indicate the presence of phthalates in the plastic material used to transfer of the sample for analysis and mainly the use of silicone lubricants on glass cuts.

These results were corroborated by NMR spectroscopic data (Table 2) and their structure, including the numbering of atoms, is shown in Figure 3.

Table 2. NMR spectroscopic data ^a for the compounds present in M8R sub-fraction isolated from *S. spinosum*.

L ^b	Stachydrine				n-Alkane				L ^b	Rutin	
	¹³ C NMR	¹ H NMR	¹³ C NMR	¹ H NMR	¹³ C NMR	¹ H NMR	¹³ C NMR	¹ H NMR		¹³ C NMR	¹ H NMR
1					13.1	0.90			1	101.0	5.10
2	76.2	4.01			21.7	1.30	157.9	nt	2''	74.3	3.46
3	25.2	2.30	2.49		30.9	1.26	134.1	nt	3''	76.7	3.40
4	18.4	2.12			28.4	1.25–1.28	178.0	nt	4''	70.0	3.25
5	66.6	3.68	3.49		28.8	1.25–1.29	157.0	nt	5''	75.8	3.31
6	43.7	3.13			28.8	1.25–1.30	93.4	6.40	6''	67.1	3.37
7	51.3	3.30			28.8	1.25–1.31	164.6	nt	1'''	103.3	4.51
8	169.4	-					98.5	6.20	2'''	70.7	3.62
9							161.5	nt	3'''	70.8	3.52
10							104.2	nt	4'''	72.5	3.26
1'							121.7	nt	5'''	68.3	3.43
2'							116.2	7.66	6'''	16.5	1.10
3'							144.4	nt			
4'							148.3	nt			
5'							114.6	6.87			
6'							122.1	7.16			

^a Chemical shift in δ scale (ppm). ^b Location (structure in Figure 3). NMR spectra of M8R sub-fraction were obtained at 600 MHz in deuterated methanol (Met-d4).

Figure 3. The chemical structure of the three major compounds presents in the M8R sub-fraction.

3.3. Synthesis of Stachydrine and Its Identification

The next working step was the preparation of stachydrine from L-proline and methyl iodide. After synthesis of stachydrine as hydroiodide, its structure was identified by several analyses. Some results of analyses are shown in Table S1 and in Figure S1 (Supplementary content), which enabled the identification of the nitrogen atom in stachydrine. After synthesis and identification of stachydrine, the two other compounds identified (benzalkonium chloride and rutin) were purchased. Quantification of the compounds in Fr. 18 was performed using the synthesized stachydrine and purchased standards of rutin and benzalkonium chloride, which simultaneously repeatedly confirmed their presence in fraction. The content of these compounds in the M8R sub-fraction were as following: stachydrine—89%, benzalkonium chloride—7%, and rutin—4%.

3.4. Cytostatic Effect of the Substances Present in the Most Active Sub-Fraction

There are several components of M8R sample that can be responsible for the observed cytostatic effect. Stachydrine, the compound present in a higher concentration, had alone a weak inhibitory effect (Table 3) on the growth of HeLa cells ($IC_{50} = 70 \mu M$). Benzalkonium

chloride showed a strong growth suppressive effect against HeLa cells ($IC_{50} = 0.8 \mu M$) and MCF-7 cells ($IC_{50} = 5 \mu M$), but unfortunately also against normal cells (same intensity). The growth suppression of HeLa and MCF-7 cells by benzalkonium chloride at a concentration of $10 \mu M$ was determined to be 88%, and 65%, respectively. The mixture of stachydrine with benzalkonium chloride at the same concentration of both compounds ($5 \mu M$) completely suppressed the growth of all tested tumor cells (Jurkat, HeLa, MCF-7, and MDA cell lines). The same effect was observed for the mixture of $1 nM$ stachydrine and $5 \mu M$ benzalkonium chloride (1:1) (Table 3).

Table 3. Survival of cells (%) at different concentrations of the compounds present in M8R subfraction isolated from *S. spinosum*.

Compound	Concentration (μM)	Jurkat	HeLa	MCF-7	MDA	3T3
Stachydrine	100	100	28.1 ± 2.2	64.2 ± 3.1	nt	89.0 ± 3.8
	10	100	81.2 ± 3.0	79.0 ± 4.2	nt	100
	1	100	89.0 ± 4.2	80.4 ± 4.3	nt	100
	0.1	100	91.2 ± 5.1	88.2 ± 4.1	nt	100
	0.01	100	95.1 ± 4.5	86.3 ± 4.3	nt	100
	0.001	100	95.0 ± 5.0	100	nt	100
Benzalkonium chloride	100	nt	nt	nt	nt	nt
	10	nt	12.1 ± 2.0	35.0 ± 2.0	nt	10.0 ± 1.8
	1	69.1 ± 3.2	43.0 ± 3.0	64.2 ± 3.1	nt	42.2 ± 2.9
	0.1	84.1 ± 4.0	81.1 ± 4.0	84.1 ± 4.2	nt	71.0 ± 3.4
	0.01	92.2 ± 5.1	79.3 ± 4.2	92.3 ± 5.0	nt	81.1 ± 3.9
	0.001	93.2 ± 5.0	79.1 ± 4.3	95.2 ± 4.5	nt	100
Rutin	100	78.6 ± 3.1	100	100	94.0 ± 4.2	96.0 ± 4.0
	10	85.9 ± 3.7	100	100	99.3 ± 5.1	100
	1	91.3 ± 4.2	100	100	98.0 ± 4.6	100
	0.1	100	100	100	98.6 ± 4.4	100
	0.01	100	100	100	99.3 ± 5.1	100
	0.001	100	100	100	100	100
Mixture of stachydrine, benzalkonium chloride and rutin (89% + 7% + 4%)		0.28 ± 0.07	76.0 ± 3.9	35.5 ± 1.6	13.5 ± 3.1	52.0 ± 3.3
Mixture of stachydrine and benzalkonium chloride ^a (in μM of individual compounds)	5 + 5	nt	nt	nt	nt	42.0 ± 3.6
	2.5 + 5	nt	nt	nt	nt	42.0 ± 5.9
	1 + 5	nt	nt	nt	nt	42.0 ± 2.1
	0.1 + 5	nt	nt	nt	nt	43.5 ± 3.3
	0.01 + 5	nt	nt	nt	nt	43.0 ± 3.4
	0.001 + 5	nt	nt	nt	nt	43.5 ± 5.1
Paclitaxel	0.1	ND	nt	6.2 ± 1.3	12.1 ± 1.9	nt
	0.01	ND	27.3 ± 2.2	31.0 ± 2.6	48.2 ± 3.1	nt
	0.001	ND	68.0 ± 3.2	60.2 ± 3.0	72.4 ± 3.3	nt
DMSO		100	100	100	100	100

^a Concentration, 10^{-4} – 10^{-9} mol/L. The data are the mean $\pm$ SD ($n = 3$). $80 \mu L$ of cells suspension + $20 \mu L$ of compound in dimethyl sulfoxide (DMSO) or in mixture of compounds: $10 \mu L$ of the both compounds (1:1) in analyses were used. ND, not determined. Paclitaxel served as positive control. DMSO (a solvent of studied compounds) was used in a final concentration 0.02%; nt—Not tested.

The IC_{50} values of Paclitaxel as positive control on HeLa, MCF-7, and MDA cells were as followed: $4.2 \mu M$, $3.8 \mu M$, and $8.6 \mu M$, respectively.

Rutin showed no inhibitory effect on cell growth of MCF-7 and HeLa at the concentrations of 10^{-4} to 10^{-9} M. Nevertheless, it showed a weak inhibitory effect against the growth of leukemic and mammary gland tumor cells.

4. Discussion

The present work shows the potential cytotoxic activity of *S. spinosum* extracts against the tested cell lines. The anti-proliferative properties of *S. spinosum* extracts were previously tested on several cancer cell lines (renal, prostate, melanoma), showing potential cytotoxic effect, which was partially attributed to the action of tormentic acid [20]. Among the four fractions studied in this work, AF was shown to be the one with the strongest anti-

proliferative activity, but overall presenting a relatively weak cytotoxic activity, with better results being obtained against human breast adenocarcinoma and lung cell lines. The results point that, after purification, the different fractions show to be more active leading to better results, than those obtained before purification.

The isolated M8R sub-fraction contained three major compounds, two of which ammonium compounds, benzalkonium chloride and stachydrine, and also a flavonoid glycoside, rutin. While all three compounds have been reported in the literature, this is the first study to identify them in *S. spinosum*.

Stachydrine is a major constituent of different herbs, plants and fruits, and it is known to possess 0.6 medicinal effects. Rathee et al. [23] reported an in vitro anti-cancer activity of stachydrine against prostate tumor cell lines (PC3 and LNCaP). Nowadays, stachydrine is used as an osmo-protectant in clinics to promote blood circulation and to ameliorate HU-VECs injury induced by anoxia-reoxygenation [24], and it is also able to suppress tubular epithelial cell apoptosis and to postpone renal intestinal fibrosis development in an animal model [25]. Stachydrine is also known to inhibit the angiotensin II-induced heart hypertrophy via blockade of the NF- κ B signal pathway [26] and to improve high-glucose induced endothelial cell senescence [27] and it is used as a marker of citrus consumption [28]. In addition to its anti-cancer action, regulation of the uterus, neuroprotective effects, etc. the pharmacokinetic properties of stachydrine are also discussed [29].

Benzalkonium chloride is a quaternary ammonium compound used as a biocide, a cationic surfactant and a phase transfer agent to preserve cosmetics, mouthwashes, and skin cleansers [30] and can also be used for cold sterilization of medical and dental instruments [31]. The results of the present work are in accordance with the conclusions of Bouchemi et al. [32] and Ammar et al. [33]. The results reported by these authors showed that benzalkonium chloride, the most commonly used preservative in topical glaucoma medications, has widely and repeatedly shown toxic and pro-inflammatory effects at the level of the ocular surface and possibly in deeper structures like the trabecular meshwork. The mixture of stachydrine and benzalkonium chloride presented a significant synergistic effect to inhibit the growth of all tumor cells tested. On the other hand, anti-proliferative activity of this mixture against non-cancer 3T3 cells was significantly weaker. The identification and observation of the synergism of cytotoxic effects of the mixture of stachydrine with benzalkonium chloride is, for the first time, mentioned in literature. The effect of benzalkonium chloride as the biocide is not surprising, but its synergistic effect with stachydrine could be used to extend the biological spectrum and enhance the efficacy of benzalkonium based disinfection products.

Rutin is a glycosylated flavonoid found in many plants and fruits [34] it is an antioxidant and can act as an inhibitor of angiogenesis with great potential for the control of some cancers. Although rutin already showed an inhibitory effect on the growth of ovarian and hepatic tumor cells [35], in the present experiments with other cell lines, rutin showed only a very weak inhibitory activity against the growth of MDA-MB-231 and Jurkat cells. Rutin also has other therapeutic properties, such as hypocholesterolemic, anti-asthmatic, and anti-inflammatory effects [36,37], and also inhibits platelet aggregation [38].

5. Conclusions

The present study demonstrated that an ethyl acetate fraction of *S. spinosum* and the three major compounds identified in it can exert a significant dose-dependent in vitro cytotoxicity especially against HeLa and MCF-7 cell lines, but also against normal cells. The mixture of stachydrine and benzalkonium chloride was shown to have a significant synergistic effect of cytotoxicity against all tested tumor cell lines. Rutin showed no inhibitory effect on the cell growth, nevertheless, it showed a weak inhibitory effect against the growth of leukemic and mammary gland tumor cells. These results provide a potent basis for further research and pharmacological activity of the isolated secondary metabolites from thorns and leaves extract will definitely give fruitful results.

Supplementary Materials: MS spectroscopic analysis of stachydrine as hydroiodide (Table S1), its ¹⁵N HMBC spectra (Figure S1), identification and NMR signal assignment of benzalkonium anion, rutine and stachydrine—Are available in Supplementary content. The following are available online at <https://www.mdpi.com/article/10.3390/app11115300/s1>, Table S1, Figure S1, Benzalkonium anion, Rutine and Stachydrine-identification and NMR signal assignment.

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