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Original article

Synthesis, screening and pro-apoptotic activity of novel acyl spermidine derivatives on human cancer cell lines

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ABSTRACT

The polyamines putrescine, spermidine, and spermine are polycationic, alkyl polyamines which play a significant role in eukaryotic cell proliferation. The polyamine metabolism and function are dysregulated in tumor cells making them an attractive therapeutic target by employing polyamine analogs. These analogs have a high degree of similarity with the structure of polyamines but not with their function. Multidrug resistance is a major factor in the failure of many chemotherapeutic drugs which necessitates further research and exploration of better novel alternatives. In the present study, Twenty-six novel acylspermidine derivatives were synthesized and evaluated for their anti-proliferative and pro-apoptotic activities on human breast cancer cells and T-lymphoblastic leukemia cells. The cell proliferation and apoptosis assays using WST-1 and annexin-V/7AAD staining respectively suggest that Compound **1** ($C_{19}H_{41}N_3O_2$), Compound **7**($C_{25}H_{51}N_3O_2$) and Compound **8** ($C_{29}H_{59}N_3O$) significantly reduced cancer cell viability in a dose- and time-dependent manner. Interestingly, compounds **7, 8** and **9** had slight or no effect on cell proliferation of non-cancerous cells. These studies speculate that these novel acylspermidine derivatives could be promising candidates in designing an anti-proliferative drug, targeting both solid and blood cancer cells.

1. Introduction

Cancer still remains the most common cause of death worldwide with the gradual increase of the incidences with age. Though initially, it responds to the chemotherapy or the radiation therapy but with the due course of time and treatment, cells acquire resistance through mutation or downregulation of the pro-apoptotic proteins and the overexpression of anti-apoptotic proteins such as bcl-2 [1,2]. Carcinogenesis involves dysregulation of the complex processes like apoptosis, angiogenesis and cell proliferation. Usage of phytochemicals or the synthetic analogs of the metabolites involved in the cell proliferation is on the rise for the treatment of the cancerous lesions triggering apoptosis. Many anticancer chemotherapeutic drugs have been developed which induce apoptosis [3,4].

Polyamines are positively charged molecules which have a crucial role in the proliferation and normal growth of a cell [5]. They are known for their unique interaction with a variety of biological targets, mostly with membrane phospholipids and

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nucleic acids through the electrostatic interactions completing the protonation of amino groups at physiological pH [6]. Spermine, spermidine, and putrescine are the natural polyamines studied widely due to the various receptors, ion channels, and the other recognition sites to which they bind [7]. Many ligands have been designed with different and specific biological targets by insertion of suitable pharmacophores onto the backbone of polymethylene including the adjustment of the position of the amine which can optimize the selectivity and affinity of the receptor [2,5,8].

During the recent years, many compounds of polyamine backbone have been synthesized which demonstrated anti-proliferative effect against diverse malignant tumors. Synthetic polyamine analogs like bisnaphthalimido or N1, N11-diethyl norspermine against breast cancer are significant with regard to anti-neoplastic activities against many solid tumor models of humans [9,10].

Many of the anticancer drugs that are widely clinically used today have high risks of adverse side effects and poor specificity and selectivity against tumors. Hence, there arises an urgent need to encounter this problem by improving the efficacy, selectiveness, and safety of the anti-tumor agents which can be done by modification with specific polyamine motifs [7] elevating the affinity for tumor cells and reach the biological targets. Therefore, we synthesized novel conjugates of acyl polyamines with fatty acyl group as a primary polyamine motif on the polyamine backbone. It has been hypothesized by many researchers that breast cancer is one of the most chemo-sensitive solid tumors, most of the initially responsive tumors regress and then develop resistance to a broad spectrum of drugs [11,12].

Moreover, the human breast cancer cell line MCF-7 is considered as the most common malignant type of breast cancer used widely around the laboratories as a tumor model in screening to study the anticancer effects and also in the investigation of the mechanism of action of hormones [13–15]. Additionally, the T-lymphoblastic leukemia cell line Jurkat is also a documented experimental model for hematological cancers in the screening of the anticancer activity as well as the cellular and molecular mechanisms of drugs exhibiting anticancer proprieties [16–18].

The aim of this study was to evaluate the anti-proliferative and pro-apoptotic effects of synthetic acylspermidines on the human adenocarcinoma cell line MCF-7 and the T-lymphoblastic leukemia cell line Jurkat.

2. Material and methods

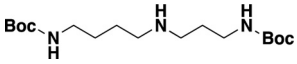
2.1. The general scheme of synthesis of active compounds

The general scheme of synthesis of the compounds is depicted in Fig. 1.

2.2. Synthesis of N^4-(3-hydroxylauroyl) spermidine (compound 1)

2.2.1. N^1,N^8-bis-tert-butoxycarbonylspermidine

2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (4.85 g, 19.7 mmol) in dry THF (25 mL) was added dropwise to a solution of spermidine (1.36 g, 9.36 mmol) and triethylamine (3.91 mL, 28.1 mmol) in dry THF (25 mL) with cooling in an ice bath and then stirred overnight at room temperature. After the reaction, the solvent was removed in vacuo, and 1 M NaOH (50 mL) was added to the resultant residue. The aqueous layers were extracted with dichloromethane (50 mL × 3). The combined organic layers were washed with saturated aqueous sodium chloride (50 mL), dried over Na_2SO_4 and concentrated *in vacuo* to give white solid, which was then recrystallized with *n*-hexane/dichloromethane to give white solid of N^1,N^8-bis-*tert*-butoxycarbonylspermidine (2.58 g, 7.47 mmol, 79.8%).



1H NMR (500 MHz, $CDCl_3$) δ 5.13 (s, 1H, NHCO), 4.80 (s, 1H, NHCO), 3.27–3.15 (m, 2H, $C\underline{H}_2NHCO$), 3.14–3.02 (m, 2H, $C\underline{H}_2NHCO$), 2.65 (t, J = 6.6 Hz, 2H, $(C\underline{H}_2)_4NHC\underline{H}_2$), 2.59 (t, J = 6.6 Hz, 2H, $(CH_2)_3NHC\underline{H}_2$), 1.72–1.58 (m, 2H, $NHCH_2C\underline{H}_2CH_2NHCO$), 1.58–1.46 (m, 4H, $NHCH_2C\underline{H}_2C\underline{H}_2CH_2NHCO$), 1.42 (s, 18H, $C(CH_3)_3$).

2.2.2. N^1, N^8-bis-tert-butoxycarbonyl-N^4-(3-hydroxylauroyl) spermidines

Under nitrogen atmosphere, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (665 mg, 3.47 mmol) was added to a solution of N^1, N^8-bis-*tert*-butoxycarbonylspermidine (1.00 g, 2.89 mmol), 3-hydroxylauric acid (626 mg, 2.89 mmol) and dimethylamino pyridine (424 mg, 3.47 mmol) in dichloromethane (20 mL), and stirred for 2 days at room temperature. Then a solution of 10% (w/w) citric acid (10 mL) was added to the reaction

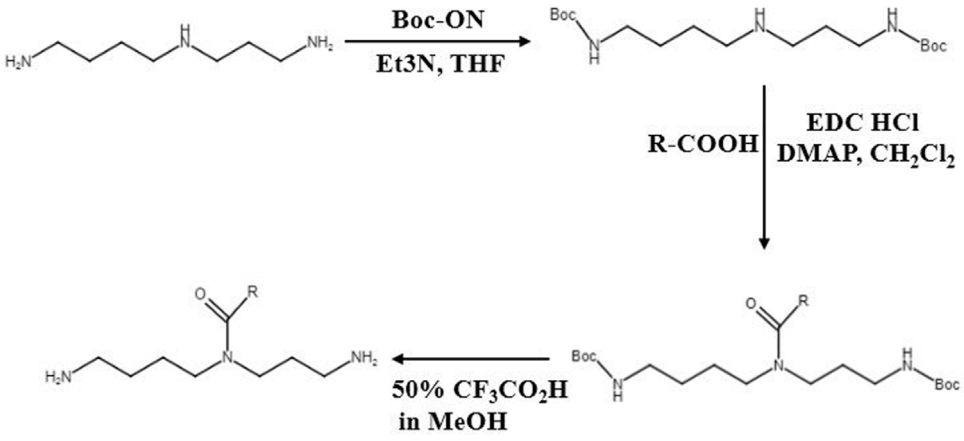
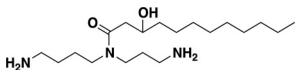


Fig 1. Overview of synthesis of active compounds. Compound **1**: (R = -CH_2-CH(OH)-$(CH_2)_7$-CH_3); Compound **7**: (R = -CH_2-$(CH_2)_5$-CH_2-CH = CH-CH_2-CH(OH)-$(CH_2)_5$-CH_3); Compound **8**: (R = -CH_2-$(CH_2)_9$-CH_2-CH = CH-CH_2-$(CH_2)_7$-CH_3); Compound **9**:(R = -CH_2-$(CH_2)_6$-CH_2-CH = CH-CH_3).

mixture and stirred for 10 min. The reaction mixture was extracted with dichloromethane (10 mL × 3), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 1:1) to give N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(3-hydroxylauroyl) spermidine as a colorless oil (1.03 g, 1.91 mmol, 66.1%), which was sufficiently pure for further use.

2.2.3. N^4-(3-hydroxylauroyl) spermidine

Under nitrogen atmosphere, N^1, N^8-bis-*tert*-butoxycarbonyl-N^4- (3-hydroxylauroyl) spermidine (950 mg, 1.74 mmol) was dissolved into 50% (v/v) trifluoroacetic acid solution in dichloromethane (10 mL) and stirred for 20 min. After the addition of methanol (30 mL), the solvent was removed *in vacuo*. Then 1 M NaOH (10 mL) was added, and the aqueous solution was extracted with dichloromethane (10 mL × 3). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give N^4-(3-hydroxylauroyl) spermidine as a colorless oil (535 mg, 1.56 mmol, 54.0%).



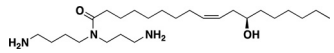
2.3. Synthesis of N^4-(ricinoleoyl) spermidine (compound 7)

*2.3.1. N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(ricinoleoyl) spermidines*

Under nitrogen atmosphere, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (230 mg, 1.20 mmol) was added to a solution of N^1, N^8-bis-*tert*-butoxycarbonylspermidine (346 mg, 1.00 mmol), ricinoleic acid (299 mg, 1.00 mmol) and dimethylamino pyridine (146 mg, 1.20 mmol) in dichloromethane (6 mL), and stirred for 2 days at room temperature. Then a solution of 10% (w/w) citric acid (3 mL) was added to the reaction mixture and stirred for 10 min. The reaction mixture was extracted with dichloromethane (5 mL × 3), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 1:1) to give N^1, N^8-bis- *tert*-butoxy carbonyl-N^4-(ricinoleoyl) spermidine (198 mg, 0.316 mmol, 31.6%) as a colorless oil, which was sufficiently pure for further use.

2.3.2. N^4-(ricinoleoyl)spermidine

Under nitrogen atmosphere, N^1, N^8-bis-Boc-N^4-(ricinoleoyl) spermidine (198 mg, 0.316 mmol) was dissolved into 50% (v/v) trifluoroacetic acid solution in dichloromethane (3 mL) and stirred for 20 min. After the addition of methanol (30 mL), the solvent was removed *in vacuo*. Then 1 M NaOH (3 mL) was added, and the aqueous solution was extracted with dichloromethane (3 mL × 3). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give N^4-(ricinoleoyl) spermidine as a pale-yellow oil (132 mg, 0.310 mmol, 98.1%).



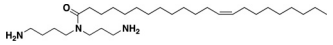
2.4. Synthesis of N^4-(erucoyl) spermidine (compound 8)

*2.4.1. N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(erucoyl) spermidines*

Under nitrogen atmosphere, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (115 mg, 0.60 mmol) was added to a solution of N^1, N^8-bis-*tert*-butoxycarbonylspermidine (173 mg, 0.502 mmol), erucic acid (170 mg, 0.502 mmol) and dimethylamino pyridine (73 mg, 0.60 mmol) in dichloromethane (3 mL), and stirred for 2 days at room temperature. Then a solution of 10% (w/w) citric acid (3 mL) was added to the reaction mixture and stirred for 10 min. The reaction mixture was extracted with dichloromethane (3 mL × 3), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 1:1) to give N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(erucoyl) spermidine (297 mg, 0.447 mmol, 89.4%) as a colorless oil, which was sufficiently pure for further use.

2.4.2. N^4-(erucoyl)spermidine

Under nitrogen atmosphere, N^1, N^8-bis-Boc-N^4-(erucoyl) spermidine (297 mg, 0.447 mmol) was dissolved into 50% (v/v) trifluoroacetic acid solution in dichloromethane (3 mL) and stirred for 20 min. After the addition of methanol (10 mL), the solvent was removed *in vacuo*. Then 1 M NaOH (3 mL) was added, and the aqueous solution was extracted with dichloromethane (3 mL × 3). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give N^4-(erucoyl) spermidine as a pale-yellow oil (204 mg, 0.437 mmol, 97.7%).



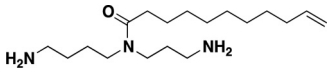
2.5. N^4-(10-undecenoyl) spermidine (compound 9)

*2.5.1. N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(10-undecenoyl) spermidines*

Under nitrogen atmosphere, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (152 mg, 0.79 mmol) was added to a solution of N^1, N^8-bis-*tert*-butoxycarbonylspermidine (228 mg, 0.662 mmol), 10-undecenoic acid (122 mg, 0.662 mmol) and dimethylamino pyridine (96 mg, 0.79 mmol) in dichloromethane (3 mL), and stirred for 2 days at room temperature. Then a solution of 10% (w/w) citric acid (3 mL) was added to the reaction mixture and stirred for 10 min. The reaction mixture was extracted with dichloromethane (3 mL × 3), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 1:1) to give as a colorless oil N^1, N^8-bis-*tert*-butoxycarbonyl-N^4-(10-undecenoyl) spermidine (302 mg, 0.590 mmol, 89.4%), which was sufficiently pure for further use.

2.5.2. N^4-(10-undecenoyl) spermidines

Under nitrogen atmosphere, N^1, N^8-bis-Boc-N^4-(10-undecenoyl) spermidine (302 mg, 0.590 mmol) was dissolved into 50% (v/v) trifluoroacetic acid solution in dichloromethane (3 mL) and stirred for 20 min. After the addition of methanol (10 mL), the solvent was removed *in vacuo*. Then 1 M NaOH (3 mL) was added, and the aqueous solution was extracted with dichloromethane (3 mL × 3). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give N^4-(10-undecenoyl) spermidine as a pale-yellow oil (182 mg, 0.584 mmol, 99.9%).



2.6. Preparation of stock solution and dilution

The Stock solutions of the drugs with 100 mM concentration were prepared in DMSO (SIGMA-ALDRICH, USA, catalog no. D8418) for compound **7** and **8.** While the remaining compounds were prepared in tissue culture grade sterile water (Invitrogen™ USA, catalog no. 15230-204) and stored at −20 °C. Working standard dilutions to 1 mM were done in tissue culture grade sterile water and kept at 4 °C. For each experiment, fresh dilution of the drug

Table 1
Molecular structures and formulae of acylspermidine derivatives.

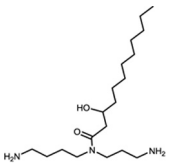
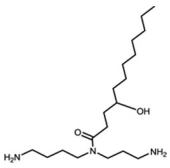
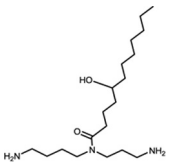
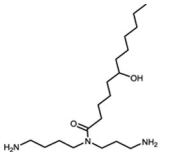
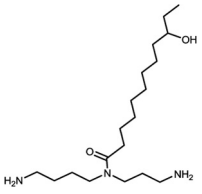
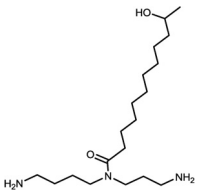
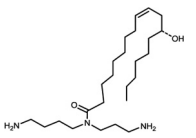
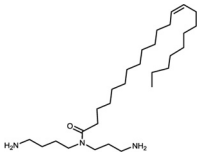
Compound	Molecular formula	Structure
1	$C_{19}H_{41}N_3O_2$	
2	$C_{19}H_{41}N_3O_2$	
3	$C_{19}H_{41}N_3O_2$	
4	$C_{19}H_{41}N_3O_2$	
5	$C_{19}H_{41}N_3O_2$	
6	$C_{19}H_{41}N_3O_2$	
7	$C_{25}H_{51}N_3O_2$	
8	$C_{29}H_{59}N_3O$	
9	$C_{18}H_{37}N_3O$	

Table 1 (*Continued*)

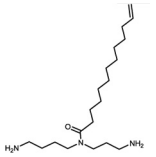
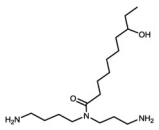
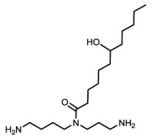
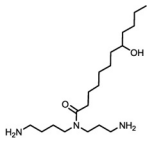
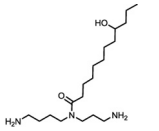
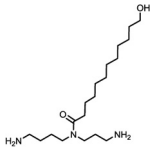
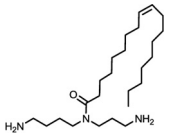
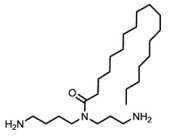
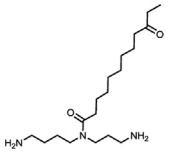
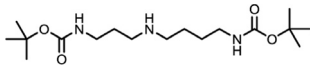
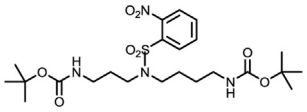
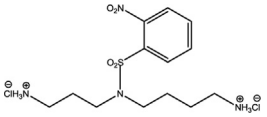
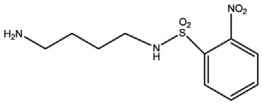
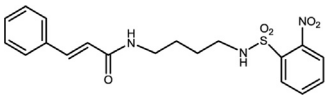
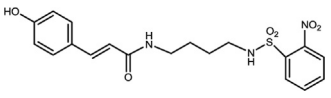
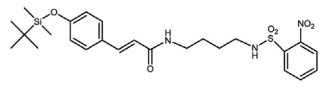
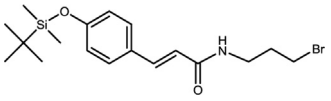
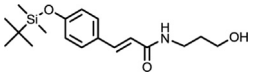
Compound	Molecular formula	Structure
		
10	$C_{17}H_{37}N_3O_2$	
11	$C_{19}H_{41}N_3O_2$	
12	$C_{19}H_{41}N_3O_2$	
13	$C_{19}H_{41}N_3O_2$	
14	$C_{19}H_{41}N_3O_2$	
15	$C_{25}H_{51}N_3O$	
16	$C_{25}H_{53}N_3O$	
17	$C_{19}H_{39}N_3O_2$	
18	$C_{17}H_{35}N_3O_4$	
19	$C_{23}H_{38}N_4O_8S$	

Table 1 (*Continued*)

Compound	Molecular formula	Structure
		
20	$C_{13}H_{24}Cl_2N_4O_4S$	
21	$C_{10}H_{15}N_3O_4S$	
22	$C_{19}H_{21}N_3O_5S$	
23	$C_{19}H_{21}N_3O_6S$	
24	$C_{25}H_{35}N_3O_6SSi$	
25	$C_{18}H_{28}BrNO_2Si$	
26	$C_{18}H_{29}NO_3Si$	

was used in treatment and for all the experiments where DMSO was used as a solvent, the concentration of DMSO was maintained at 0.1- 0.5%.

2.7. Cell culture and treatment

The human breast adenocarcinoma cell line (MCF-7) and human lymphocytic leukemia cell line (Jurkat) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a humidified incubator with 5% CO_2 at 37 °C. MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (UFC biotech, Riyadh, KSA, catalog no. T-DMEM-111014) and Jurkat cells were cultured in RPMI 1640 (UFC biotech, Riyadh, KSA, catalog no. TRPMI-11111). Both DMEM and RPMI were supplemented with 10% FBS (Lifetech, catalog no. 16000-044) and 1% penicillin-streptomycin antibiotics (100 units/mL, Lifetech, catalog no. 15140-122). Normal human dermal fibroblasts (NHDFs) (catalog no. PCS-201-012) were procured from the ATCC and grown in fibroblast basal media (ATCC, catalog no. PCS 201-030) supplemented with fibroblast low kit growth serum (ATCC, catalog no. PCS 201-041) and penicillin-streptomycin antibiotics (100 units/mL, Lifetech, catalog no. 15140-122).

2.8. Cell viability assay

The effect of the acylspermidine derivatives on proliferation/inhibition was analyzed by a colorimetric cell proliferation assay using WST-1 reagent (Sigma-Aldrich, USA, catalog no. 11644807001). Briefly, the cells were seeded in 96-multiwell plates at a density of 10^4 cells/well (counted using Scepter 2.0 Handheld Automated Cell Counter, Millipore®, Billerica, MA, catalog no. PHCC20040) for MCF-7, fibroblasts or 4×10^4/well for Jurkat cells. After 24 h of incubation, the cells were exposed to different concentrations (5, 10, 30 and 50 μM) of acyl spermidines for the desired time periods. Cell proliferation rate was evaluated through a rapid WST-1 reagent. After incubation for the above-mentioned time duration, 10 μL of the WST-1 solution was added and incubated for an additional 4 h at 37 °C. Finally, the absorbance was read at 450 nm with a microplate ELISA reader (ELx800™ Biotek, USA) and the results were analyzed by the Gen5 software (Biotek, USA). Thymoquinone (Sigma-Aldrich, USA, catalog no. 274666-5G) was used as a positive control. The percentage of cell viability was calculated by assuming control (untreated) samples as 100% viable [19]. The IC50 (50% inhibitory concentration) values were calculated from the dose-response curves.

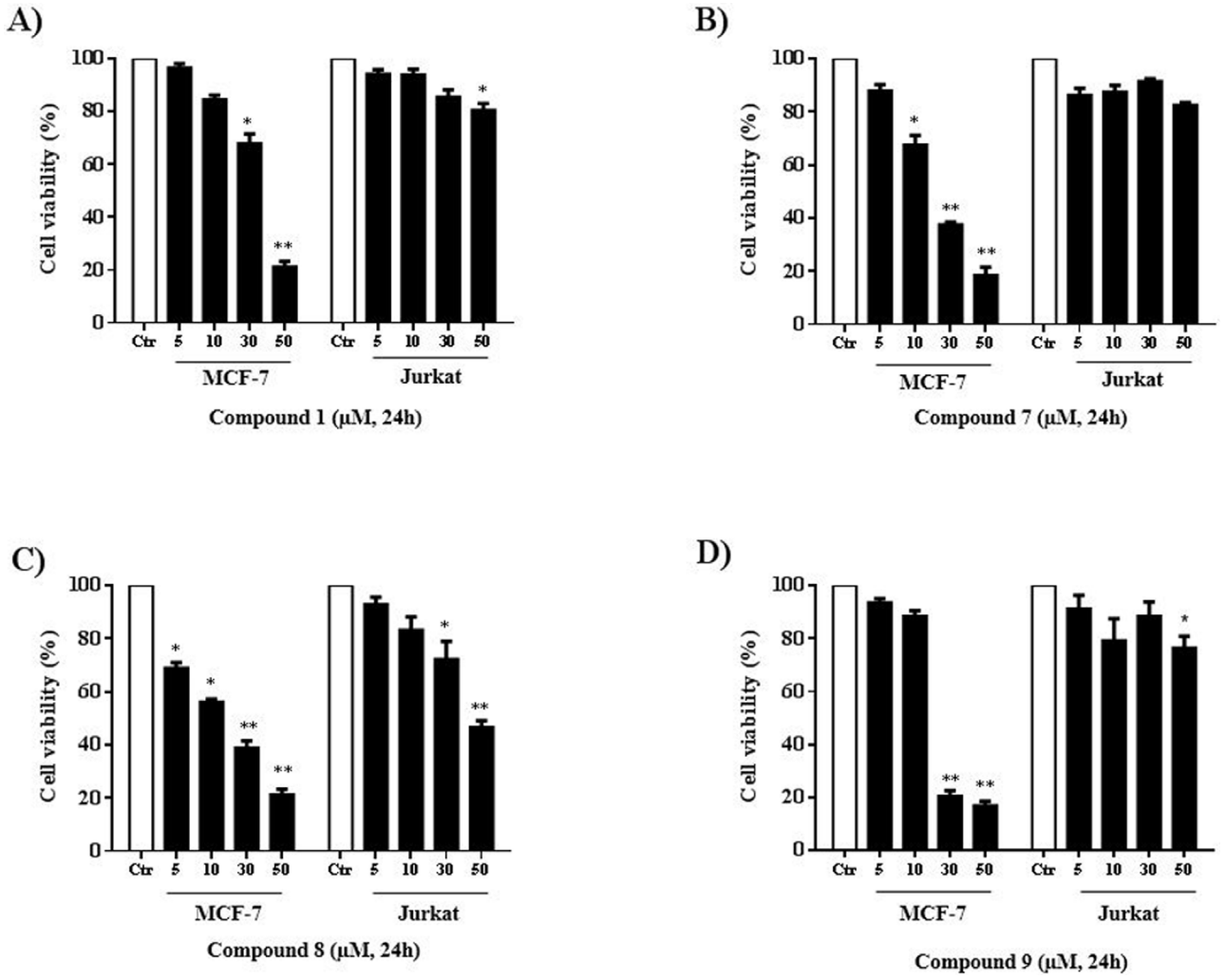


Fig. 2. Compounds **1**, **7**, **8** and **9** reduces the cell viability in a concentration-dependent way in cancer cells. MCF-7 and Jurkat cells were treated with several concentrations of compound **1** (A), compound **7** (B), compound **8** (C) and compound **9** (D) for 24 h. Cell viability rate was assessed using WST-1 assay. Represented values are mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired '*t*' test, two-tailed): *, $p < 0.05$; **, $p < 0.01$; (versus the corresponding untreated group).

Table 2
IC_{50} values (μM) of the active compounds against tumor cells are presented as mean ± S.E.M.

Compound	MCF-7	JURKAT
1	35.37 ± 2.1	56.71 ± 5.4
7	21.25 ± 2.5	61.78 ± 4.1
8	23.22 ± 1.7	33.17 ± 0.7
9	22.41 ± 5.5	56.29 ± 1.0
Thymoquinone	16.75 ± 6.3	18.91 ± 3.7

2.9. Apoptosis assay

MCF7 (2×10^5 cells/well in 6 well plate) and Jurkat cells (4×10^4 cells/well in 96 well plate) were cultured overnight and treated with different concentrations of the tested compounds. After treating the cells with desired concentration of the drugs for several time periods, Annexin V/7AAD staining was carried out as per the manufacturer's protocol. Briefly, 100 μL of the nexin reagent (Millipore®, Billerica, MA, catalog no. 4500-0450) staining solution (containing annexin V-fluorescein and 7AAD) was added and incubated at room temperature for 20 min in the dark surroundings. The forward and side scatter were recorded at 10,000 events and the subsequent percentage of the early, and the late apoptotic cells were analyzed using the Guava® easyCyte 12HT Benchtop Flow Cytometer (Millipore®, Billerica, MA), and the results plotted using the InCyte™ software (Millipore®, Billerica, MA).

2.10. Statistical analysis

All the data were presented as Mean ± SEM (standard error of the mean) of triplicates done in the same experiment or an average of at least three independent experiments. The differences between the control and the treated groups for cell proliferation and apoptosis were analyzed by Student's *t*-test (two-tailed) using GraphPad Prism 6 (Graph Pad Software, SanDiego, USA) and the significant differences were indicated as $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ (Table 2).

3. Results

3.1. NMR data of the active compounds

Compound 1: (Yield 54%) 1H NMR (500 MHz, $CDCl_3$) δ 3.99 (m, 1H, C<u>H</u>(OH)), 3.46–3.25 (m, 4H, CH_2NCH_2), 2.76–2.54 (m, 4H, $2 \times C\underline{H}_2NH_2$), 2.50–2.32 (m, 2H,$COC\underline{H}_2$), 1.70–1.50 (m, 6H, $NCH_2\underline{C}H_2\underline{C}H_2CH_2NH_2$, $NCH_2\underline{C}H_2\underline{C}H_2NH_2$), 1.45 (m, 2H, CH(OH) $C\underline{H}_2CH_2$), 1.26 (m, 14H, $(C\underline{H}_2)_7CH_3$), 0.88 (t, J = 7.0 Hz, 3H, CH_3).

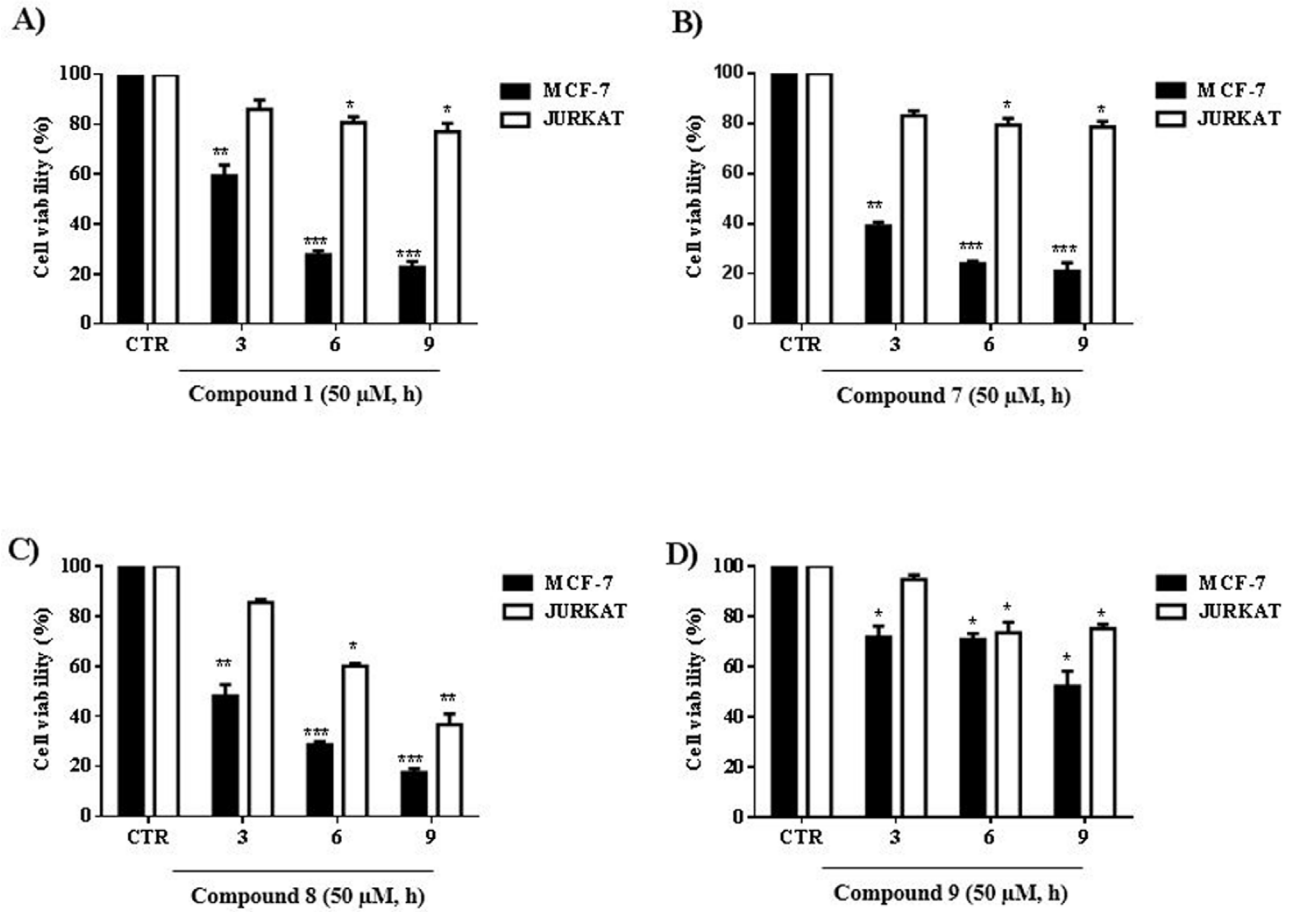


Fig. 3. Compounds **1**, **7**, **8** and **9** reduces cell viability in a time-dependent manner in cancer cells. MCF-7 and Jurkat cells were treated with 50 μM of the compound 1 (A), compound **7** (B), compound **8** (C) and compound **9** (D) for several time durations. Cell viability rate was assessed using WST-1 assay. Values are mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired '*t*' test, two-tailed): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus untreated group).

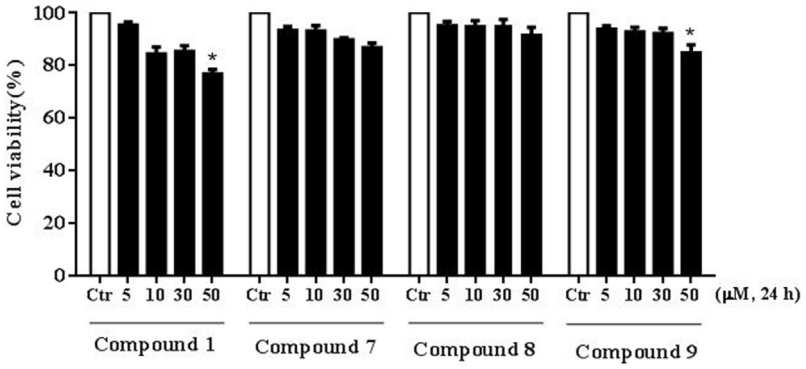


Fig. 4. Compounds **1**, **7**, **8** and **9** exhibit slight or no cytotoxicity on normal Fibroblasts. NHDFs (normal human dermal fibroblasts) were treated with several concentrations of compound **1**, compound **7**, compound **8** and compound **9** for 24 h. Cell viability rate was assessed using WST-1 assay. Represented values are mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired 'test, two-tailed): *, $p < 0.05$ (versus the corresponding untreated group).

Compound 7: (Yield 98.1%) 1H NMR (500 MHz, $CDCl_3$) δ 5.63–5.33 (m, 2H, CH = CH), 3.71–3.50 (m, *J* = 6.0 Hz, 1H, C<u>H</u>(OH)), 3.50–3.17 (m, 4H, CH_2NCH_2), 2.82–2.57 (m, 4H, 2 × $C\underline{H}_2NH_2$), 2.41–2.23 (m, 2H, $COCH_2$), 2.20 (t, *J* = 6.6 Hz, 2H, $CHC\underline{H}_2CH(OH)$), 2.04 (q, *J* = 6.9 Hz, 2H, $CHC\underline{H}_2CH_2$), 1.87–1.51 (m, 10H, $NCH_2C\underline{H}_2C\underline{H}_2CH_2NH_2$, $NCH_2C\underline{H}_2CH_2NH_2$, $COCH_2C\underline{H}_2$ $CH(OH)C\underline{H}_2CH_2$), 1.51–1.19 (m, 16H, $(CH_2)_4$, $(CH_2)_4$), 0.88 (t, *J* = 6.9 Hz, 3H, CH_3).

Compound 8: (Yield 97.7%) 1H NMR (500 MHz, $CDCl_3$) δ 5.40–5.29 (m, 2H, CH = CH), 3.49–3.15 (m, 4H, CH_2NCH_2), 2.80–2.60 (m, 4H, 2 × $C\underline{H}_2NH_2$), 2.35–2.22 (m, 2H, $COCH_2$), 2.00 (q, *J* = 6.4 Hz, 4H, $C\underline{H}_2CH=CHC\underline{H}_2$), 1.86–1.40 (m, 8H, $NCH_2C\underline{H}_2C\underline{H}_2CH_2NH_2$, $NCH_2C\underline{H}_2CH_2NH_2$, $COCH_2C\underline{H}_2$), 1.37–1.11 (m, 28H, $COCH_2CH_2(C\underline{H}_2)_8$, $CH_3(C\underline{H}_2)_6$), 0.87 (t, *J* = 6.9 Hz, 3H, CH_3).

Compound 9: (Yield 99.9%) 1H NMR (500 MHz, $CDCl_3$) δ 5.78 (ddt, *J* = 20.1, 6.9, 6.3 Hz, 1H, C<u>H</u> = CH_2), 4.96 (dd, *J* = 17.2, 1.7 Hz, 1H, 1/2CH = C<u>H</u>$_2$), 4.90 (dd, *J* = 10.0, 1.4 Hz, 1H, 1/2CH = C<u>H</u>$_2$), 3.47–3.16 (m, 4H, CH_2NCH_2), 2.79–2.59 (m, 4H, 2 × $C\underline{H}_2NH_2$), 2.35–2.20 (m, 2H, $COCH_2$), 2.01 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH=CH_2$), 1.73–1.48 (m, 8H, $NCH_2C\underline{H}_2C\underline{H}_2CH_2NH_2$, $NCH_2C\underline{H}_2CH_2NH_2$, $COCH_2C\underline{H}_2$), 1.48–1.07 (m, 10H, $(CH_2)_5$).

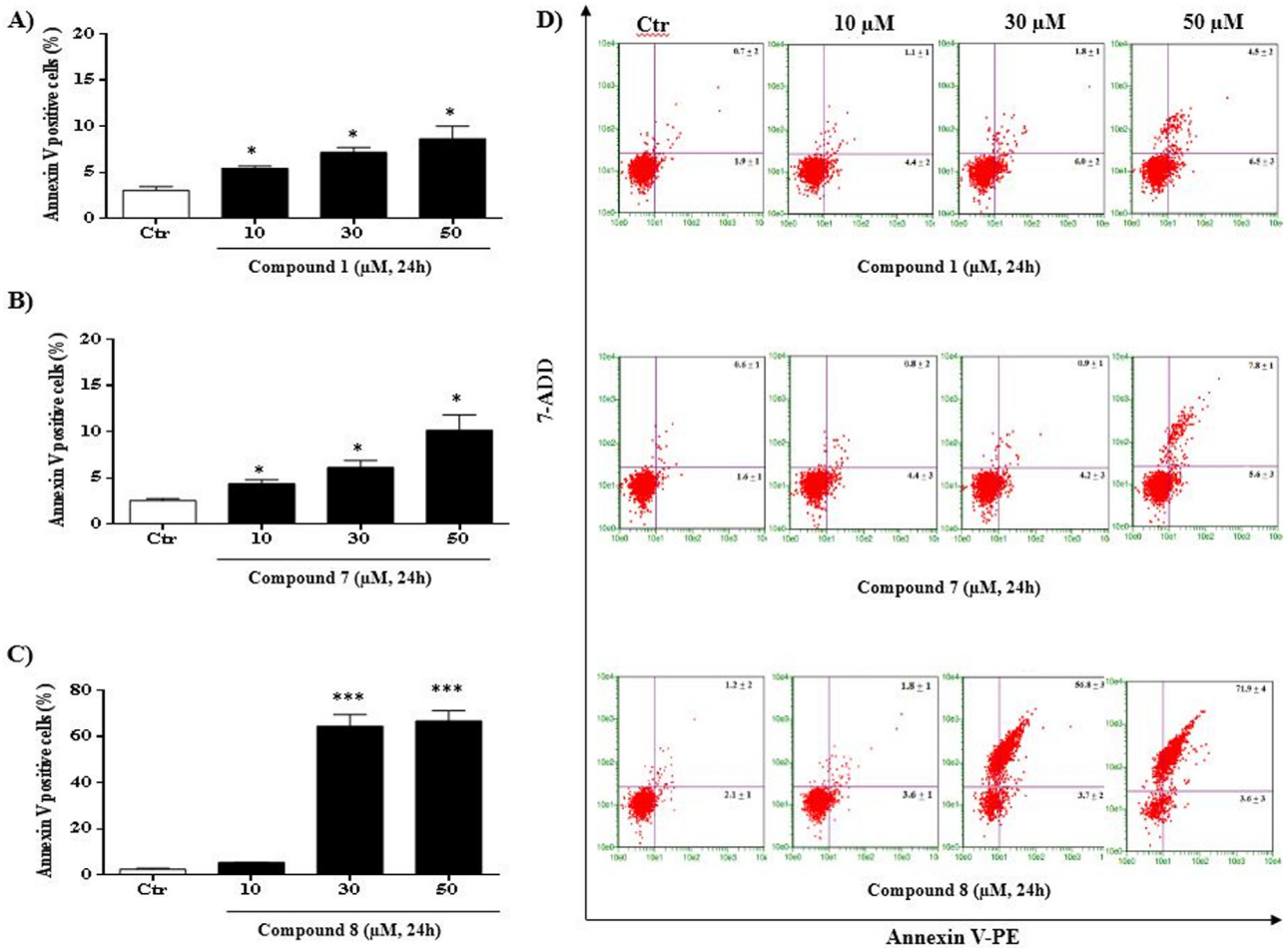


Fig. 5. Compounds **1**, **7** and **8** induce concentration-dependent apoptosis in Jurkat cells. Cells were exposed to increasing concentrations of compound **1** (A), compound **7** (B) and compound **8** (C) for 24 h. Apoptosis in Jurkat cells was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay (D). Values are Mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired 'test, two-tailed): *, $p < 0.05$; ***, $p < 0.001$ (versus untreated group).

3.2. Compounds 1, 7, 8, and 9 decreased cell viability of cancer cells by dose-dependent mechanism

Initially, we screened the 26 derivatives of acyl spermidines on MCF-7 cells to assess the anti-proliferative activity of the compounds (Table 1). The findings have shown that compounds **1**, **7**, **8** and **9** significantly decreased MCF-7 cell viability (15%, 12%, 21% & 13% for **1**, **7**, **8** and **9** respectively at highest concentration) in a concentration-dependent (5, 10, 30 and 50 μM) manner (Fig. 2, (Supplementary Figs. S1–S3 in the online version at DOI: 10.1016/j.biopha.2017.06.019)). The corresponding IC_{50} values were calculated as depicted in Table 2. The most active compound on MCF-7 was Compound **7** with an IC_{50} of 21.25 ± 2.5 μM followed by compound **9**, **8** and **1** with IC_{50} values of 22.41 ± 5.5, 23.22 ± 1.7 and 35.37 ± 2.1 μM respectively (Fig. 2 and Table 2). Unlike MCF-7 cells, compound **8** was the most active compound against Jurkat cells with IC_{50} value of 33.17 ± 0.7 μM, followed by compound **9**, **1** and **7** with IC_{50} values of 56.29 ± 1.0, 56.71 ± 5.4 and 61.78 ± 4.1 μM respectively. Thymoquinone was used as a positive control which showed significant decrease in the cell viability with an IC_{50} value of 16.75 ± 6.3 μM on MCF-7 and 18.91 ± 3.7 μM on Jurkat cells respectively (Table 2).

Thereafter, compounds **1**, **7**, **8** and **9** were selected for further cytotoxic studies on MCF-7 and Jurkat cells. Altogether, this data indicates that compounds **1**, **7** and **9** target predominantly MCF-7 cells relative to Jurkat cells and compound **8** could be a selective inhibitor of cell proliferation of both MCF-7 cells and Jurkat cells.

3.3. The cell viability inhibition induced by compounds 1, 7, 8 and 9 was time-dependent

We next investigated the time-dependent effect of the compounds **1**, **7**, **8** and **9** at a concentration of 50 μM for different time periods (3, 6 and 9 h). Incubation of MCF-7 cells with 50 μM of compounds **1**, **7** and **8** induced a time-dependent decrease in cell viability with a significant visual effect after 3 h (Fig. 3A–C). However, the decline in the cell viability of MCF-7 after exposure to compound **9**, showed a significant effect at 9 h with a cell viability of 55% (Fig. 3D). Compared to compounds **1**, **7** and **9**, compound **8** was more active against Jurkat cells in decreasing the cell viability (Fig. 3). Indeed, Compound **9** decreased the cell viability of Jurkat cells by about 60% and 40% after an incubation of 6 h and 9 h respectively (Fig. 3D). Such effect was not observed in Jurkat cells by the Compounds **1**, **7** and **9** under similar conditions. Altogether, these findings indicate that compounds **1**, **7** and **8** target predominantly MCF-7 cells relative to Jurkat cells and compound **8** could target both solid and blood tumors.

3.4. Compounds 1, 7, 8 and 9 demonstrated minimal cytotoxicity towards non-cancerous cells

To investigate the selectivity of the active compounds towards the cancer cells, we treated the normal human dermal fibroblasts (NHDFs) with compounds **1**, **7**, **8** and **9** under the similar experimental conditions that were used for tumor cells (Fig. 4).

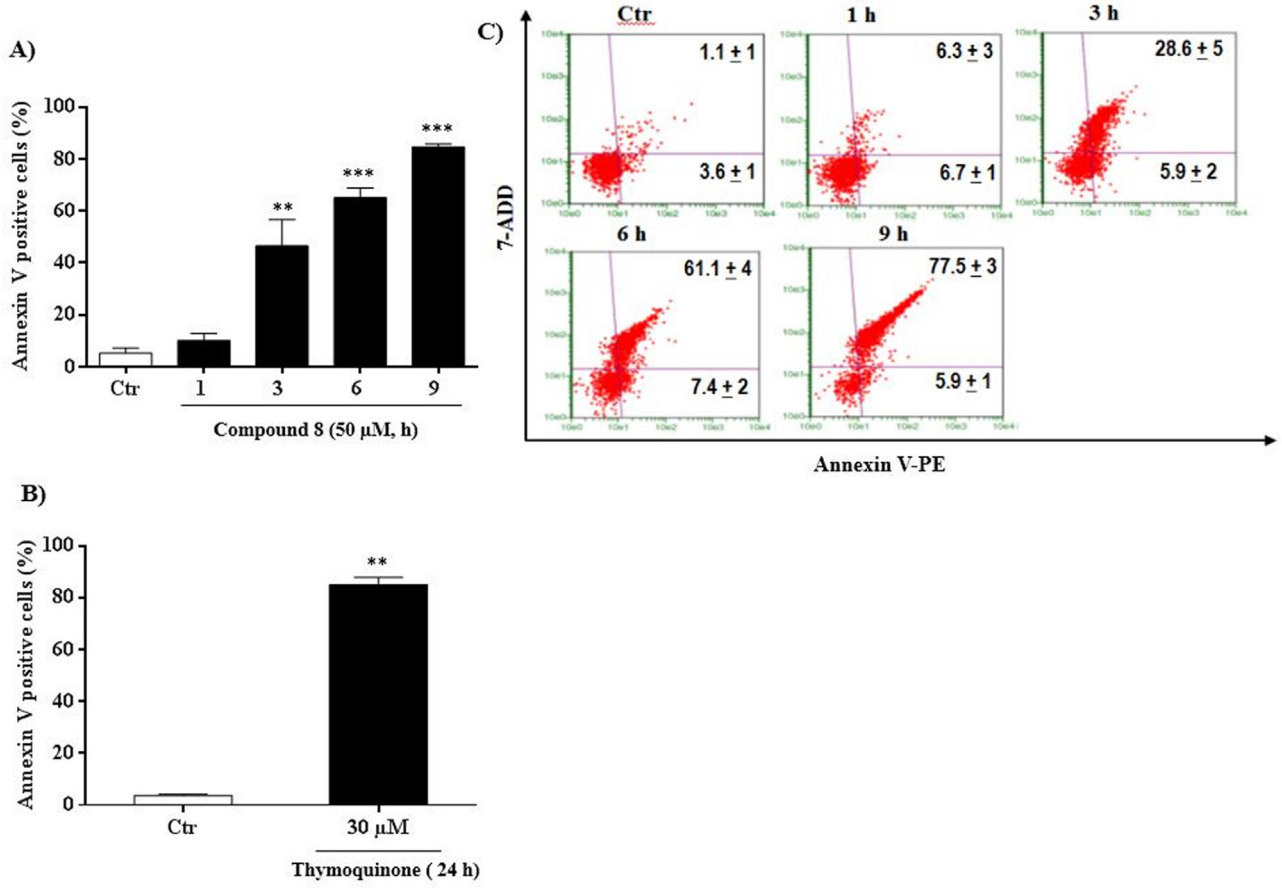


Fig. 6. Compound **8** induces a time-dependent apoptosis in Jurkat cells. Cells were treated with 50 μM of compound **8** (A) for different time durations or with 30 μM of thymoquinone (B) for 24 h. (C) Apoptosis in Jurkat cells was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay. Values are mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired 'test, two-tailed): **, $p < 0.01$; ***, $p < 0.001$ (versus untreated group).

Although, compound **1**, **7**, **8** and **9** at a concentration of 50 μM, significantly induced cell proliferation inhibition of MCF-7 cells by about 80% (Fig. 2), no such effect was found in NHDFs (Fig. 4). However, at 50 μM, compounds **1**, **7**, **8** and **9** induced slight inhibition of fibroblasts cell viability by about 23%, 13%, 8% and 15% respectively (Fig. 4) suggesting that the compound **1** showed more cytotoxicity relative to the other compounds towards NHDFs.

3.5. Compound 1, 7 and 8 induced apoptosis in MCF-7 & Jurkat cells

To determine the compound-induced mechanism(s) involved in cell viability inhibition, we studied their effect on the apoptosis in both the cancer cell lines. Annexin V-7AAD staining was carried out after treatment with either desired concentrations of compounds **1**, **7** and **8** for 24 h or with the natural anticancer compound, thymoquinone as a positive control. Fig. 5 shows that all the three compounds (**1**, **7** and **8**) were able to induce significant apoptosis in Jurkat cells, starting from 10 μM. The percentage of apoptotic cells was more pronounced with the higher concentrations (30 and 50 μM). Treating Jurkat cells using 50 μM of either compound **1** (Fig. 5A) or **7** (Fig. 5B) induced apoptosis by about 12% and 14% respectively. Interestingly, compound 8 was able to induce apoptosis in Jurkat cells by more than 70% under the same conditions (Fig. 5C). These findings indicate that compound **8** is the most active pro-apoptotic compound in Jurkat cells compared to **1** and **7** which is in agreement with the findings of cell viability (Fig. 2). To determine the chronology of the compound **8**-induced molecular and cellular events precisely, we studied its time course effects on Jurkat cells at a concentration of 50 μM. Interestingly, compound **8** induced a time-dependent apoptosis (Fig. 6). A significant increase in apoptotic cells was found already at 3 h (34.5%) and was more prominent at 6 h and 9 h by about 68% and 83.1% respectively. When Jurkat cells were incubated with 50 μM of compound **8**, it induced a time-dependent increase in annexin V-positive cells and this effect was quite significant commencing from 3 h and reached higher levels to approximately 76% at 9 h (Fig. 6A). Then, we analyzed the effect of compounds **1**, **7**, **8** on the apoptosis in MCF-7 at a concentration of 50 μM (Fig. 7). Unlike Jurkat cells, the compound **1** was the most pro-apoptotic compound among the others (Fig. 7B). While compound **7** and **8** induced apoptosis in MCF-7 by about 23% and 18% respectively, the percentage of apoptotic cells reached to 59% in compound **1**-treated MCF-7 cells (Fig. 7A and B). Altogether, these findings suggest that MCF-7 and Jurkat cells respond in different ways to compound **1**, **7** and **8** suggesting that these compounds trigger apoptosis in solid and hematological tumors by different signaling pathways.

4. Discussion

The natural polyamines are involved in multiple cellular functions. These polyamines have a pivotal role in cell growth and proliferation which makes them a promising target for anti-cancer therapy[1,20]. Thus, there is an increasing demand to develop competitive polyamine analogs which compete for uptake of natural polyamines and hence shutting down the biosynthesis of polyamines in the cell by initiating polyamine degradation [21].

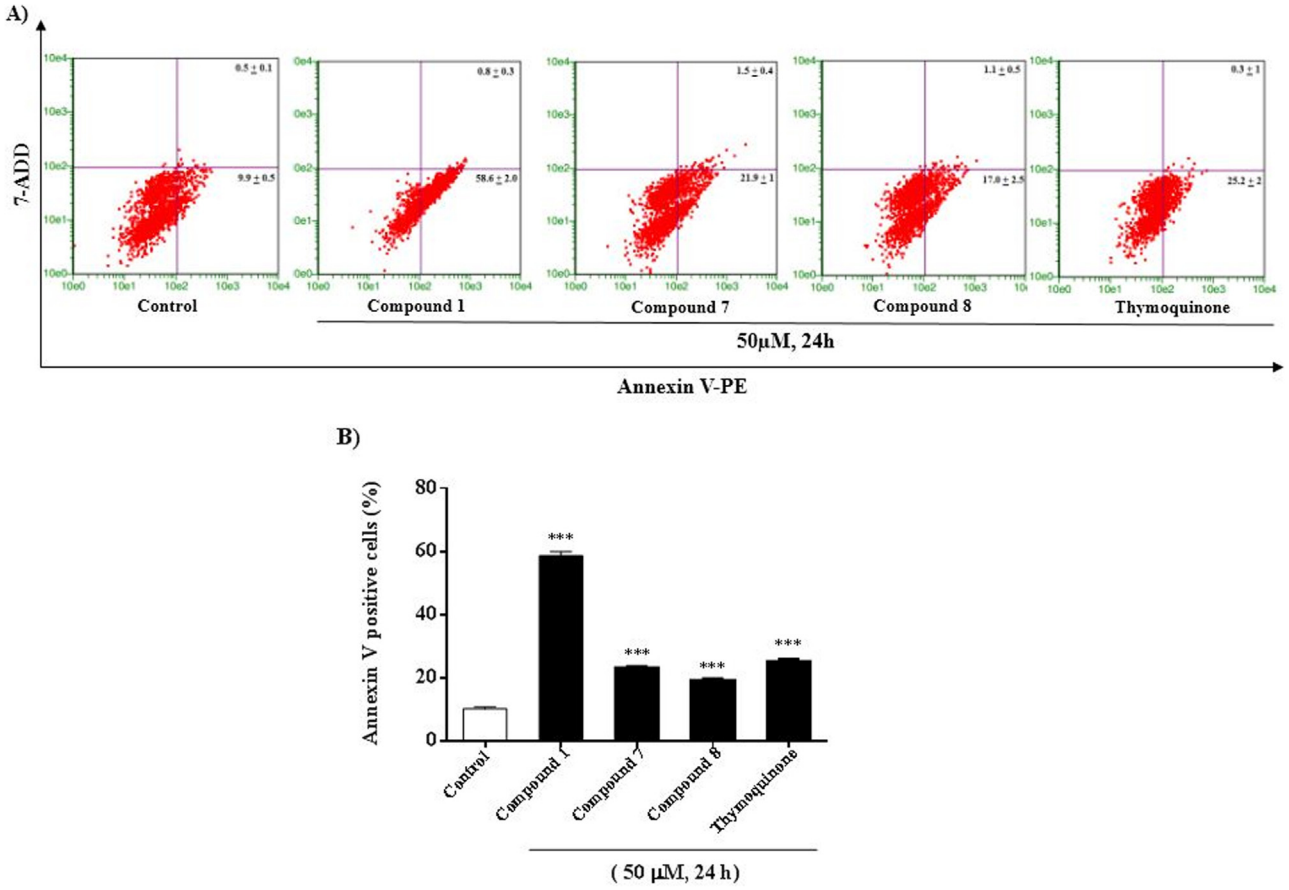


Fig. 7. Compounds **1**, **7** and **8** induces a concentration-dependent apoptosis in MCF-7cells. Cells were exposed to increasing concentrations of compound **1**, compound **7** and compound **8** for 24 h. (A) Apoptosis in MCF-7 cells was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay and the percentage of annexin-V positive cells are depicted (B). Values are mean ± S.E.M. of three experiments (n = 3); statistically significant (unpaired '*t*' test, two-tailed): *, $p < 0.05$; ***, $p < 0.001$ (versus untreated group).

The present study indicates that Compound **1**($C_{19}H_{41}N_3O_2$), Compound **7** ($C_{25}H_{51}N_3O_2$) and Compound **8** ($C_{29}H_{59}N_3O$) significantly reduced the cell viability of the human breast cancer cell line MCF-7 by dose- and time-dependent mechanisms. Unexpectedly, compounds **7** and **8** had no same effect on the apoptosis when compared to cell viability indicating that these compounds can trigger cell death mechanisms other than apoptosis. Other mechanisms including autophagy have been shown to be involved in the acylspermidine analog-induced cell proliferation inhibition [22,23]. In agreement with this hypothesis, the GC7 (N1guanyl-1,7-diamineoheptane), spermidine analog was shown to induce autophagy in Human fibrosarcoma cells [22]. Compounds **1**, **7** and **8** also reduced cell viability and induced apoptosis in Jurkat cells in a dose- and time-dependent manner. However, the anti-proliferative and pro-apoptotic activities were more pronounced with compound **8** indicating that this derivative could be a potent candidate which could target both solid and hematological tumors.

Moreover, This data shows that Compounds **1**, **7** and **8** targets predominantly MCF-7 relative to Jurkat cells. The present study is in good agreement with the recent findings that the use of polyamine analogues like N^1, N^{11}-diethylnorspermine (DENSPM) [10] and bis-amino-oxy-propyl-naphthalimido polyamine derivatives [9] against breast cancer cells and the use of hydroxylated polyamine analogs against leukemia cells [24] could be an effective measure to check the proliferation of cancer cells. Interestingly, except compound **1**, the other active compounds **7**, **8** and **9** had no or slight effect on cell viability of the NHDFs which were used as a non-cancerous cell model in this study. These observations support our conclusion that compound **8** could be a very promising inhibitor of cancer cell proliferation by targeting polyamine pathways without affecting the normal cells. Lipophilicity of the compounds plays a significant role in the transport of polyamines into the cell and the subsequent cytotoxic effect on the tumor cells. The lipophilic nature of Compound **8** is rendered by the presence of alkyl group on the side chain of the polyamine which makes it more penetrable through the cytoplasmic membrane via endocytosis. This speculation may explain its superior pro-apoptotic effects on both the cancer lines.

During the recent years, many polyamine analogs have been designed which initiate an apoptotic cascade of events in the tumors of skin, colon, breast, liver and prostate [25,26]. Phosphatidylserine (PS) is an essential phospholipid present in the inner leaflet of the plasma membrane in normal healthy mammalian cells. PS is exposed to the exterior when the cell membrane is compromised during cell death which is a hallmark of early apoptosis. This exposed PS can be stained easily using annexin V which specifically binds to it and can be detected by Flowcytometric assays. Exposure of Jurkat cells to compound **8** induced a rapid increase in the annexin V positive cells. This effect was quite significant commencing from 3 h indicating that compound **8** can decrease the cell viability of cancer cells by induction of apoptosis, but the involvement of other mechanisms is not excluded. Our findings are quite similar to a previous study wherein the bisnaphthalimido polyamines derivatives were evaluated against tumor cells [9]. Cancer cells thrive and proliferate unchecked in the polyamine-rich environments and

hence the induction of apoptosis can be the means to keep a check on the growth by siphoning off the polyamines which are required for cell growth and proliferation. Interestingly, in the present study, Compound **8** has shown inhibitory effect on tumor cell proliferation without any detrimental effect to the non-cancerous cells.

In conclusion, the present study highlights the potential of synthesized acyl spermidine derivatives in triggering apoptosis in both solid and hematological tumors. This study shows that cancer cells can respond to acylspermidine derivatives in different ways. Interestingly, most of the active compounds had no or slight inhibitory effects on normal cells. The present findings suggest that compound **8** could be a selective trigger of cell death in cancer cells and this effect could be attributed to its structure. Therefore, further studies are required to determine the signaling pathways involved in the apoptosis, induced by these synthesized derivatives.

Conflict of interest

The authors declare that they have no financial conflict of interest.

Acknowledgements

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