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Synthesis of some benzylidene thiosemicarbazide derivatives and evaluation of their cytotoxicity on U87, MCF-7, A549, 3T3 and HUVEC cell lines

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Abstract: Iron homeostasis is altered in tumours in response to a perturbation in the expression of iron-dependent proteins. Therefore, iron chelators make cancerous cells more vulnerable to iron deficiency. Compounds having thiosemicarbazide scaffold with the ability to metal complex formation have the potential to act as anticancer. A series of thiosemicarbazide derivatives were designed, synthesized successfully and their cytotoxicity was then tested on some cancerous as well as laboratory normal model systems by using colorimetric assay based on WST-1 reagent. According to the cytotoxicity results, some compounds showed high toxicity effect on both the cancerous and healthy cell lines. The results of toxicity assays on U87 and A549 cell lines showed the survivability less than 50 % at all concentrations higher than 10 ppm for all the synthesized compounds. The MCF-7 cell line exhibited approximately the same behaviour and had survivability less than 60 %. The 3T3 in compared with HUVEC cell line showed a completely different behaviour against the synthesized compounds and had survivability more than 50 %. The selectivity index was also measured and based on the study results it could be concluded that the cytotoxicity profile of the synthesized compounds on 3T3 cell line shows a significant difference, indicating a good anticancer effect of these compounds.

Keywords: *meta* and *para*-substituted benzylidene thiosemicarbazides; cytotoxicity assessment; cancerous and normal cell lines; WST-1 assay.

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INTRODUCTION

Have been known for years that cancer cells have modified metabolic pathways compared to their normal cell counterparts. For instance, there is general agreement that cancer cells, at least in a large number of solid ones, benefit from an enhancement in aerobic glycolysis over oxidative phosphorylation.[1–3] Also glutamines play a major role in the cancer cells either as a supplier for building blocks of macromolecules or through increasing the activity of the glutaminolysis pathways enhancing the ATP supply through the Krebs cycle.[4] Furthermore, while de novo fatty acid synthesis is usually suppressed in normal cells, tumour cells synthesize fatty acids at high rates.[3] NADPH can contribute to fatty acid biosynthesis as well as ROS scavenging of reactive oxygen species in tumour cells.[2,5] Tumour proliferations also need more ribose-5-phosphate, cofactors and relevant enzymes to enhance nucleotide synthesis and DNA repair.[3]

One of the different characteristics of cancer cells that can be used to design anti-cancer drugs is expression of high levels of the transferrin receptor 1 (TfR1) and internalization of iron (Fe) from transferrin (Tf) at an extraordinary rate in response to the high requirement of tumor cells for Fe.[6,7] The monoclonal antibody designated 42/6, has been reported to act as an agent with the potential to Fe deprivation through transferrin binding inhibition to its receptor, resulting in in vitro growth suppression of the human T leukemic cell line.[8]

Disorders that impair iron homeostasis are one of the most common human diseases that cancer is one of them.[9] Ribonucleotide reductase (a Fe-containing enzyme required for the conversion of ribonucleotides into deoxynucleotides for DNA synthesis) is a key enzyme in the rate limiting step of DNA synthesis.[10] It has been reported that desferrithiocin and desferrioxamine, as iron chelators, target cancerous cells in the S phase or the early S phase of the cell proliferation cycle.[10–12] Iron chelation therapy is a new strategy to cure cancers, especially those resistant, by using iron chelators depriving tumour cells from nutrient Fe.[13–15] This issue is based on the finding that the ribonucleotide reductase shows an enhanced activity in tumour cells compared to normal cells.[16–18]

Thiosemicarbazides are considered as a class of biological compounds with a wide range of activity and have been studied for their activity against viruses,[19–22] bacteria,[20,23–26] fungi[27] and cancerous cells.[20,28–30] Triapine and marboran (methisazone, Fig. 1) are two synthetic analogues of thiosemicarbazides already in market.[20] Triapine, first synthesized by Liu and co-workers[30] and still under clinical phase I/II studies,[31–33] is a more potent ribonucleotide reductase and cancer cell growth inhibitor than the antineoplastic drug, hydroxyurea.[34] Marboran is also a good anti-viral agent.[35–37] The ability of thiosemicarbazones to form chelators with metal ions, especially iron, has led researchers to synthesize derivatives of these compounds and investigate their anti-cancer effects.[38,39] As part of an ongoing research program on the synthesis of new thiosemicarbazide

derivatives as biologically active compounds, we herein report the synthesis and cytotoxicity assessment of some thiosemicarbazide derivatives on U87, MCF-7, A549, 3T3 and HUVEC cell lines.

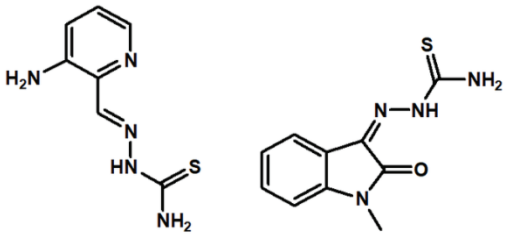


Fig. 1. Two synthetic analogues of thiosemicarbazides already in market.

EXPERIMENTAL

All chemicals, reagents and solvents were commercially available and were used without further purification. An electrothermal IA9100 melting point apparatus fixed at 1 °C/min was used to determine melting point. The IR spectra were recorded on an FT-IR Tensor 27 infrared spectrophotometer (Bruker) using KBr as a matrix. ^{1}H-NMR and ^{13}C-NMR spectra were taken by an FT-NMR Bruker Avance ultra shield Spectrometer (300 and 75 MHz in frequencies for ^{1}H and ^{13}C, respectively) using DMSO-d_6 as solvent. Also, TLC-grade silica gel G/UV 254 nm plates were used for monitoring reaction progress by the use of EtOAc/*n*-hexane (1:2 volume ratio) as eluent. Standard laboratory chemicals and a selection of reagents were obtained from the Sigma–Aldrich Company including Dulbecco's modified eagle's medium F12 (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (100 μg/ml), and phosphate-buffered saline (PBS), trypan blue dye solution, and trypsin- EDTA solution. 2-(2--methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium. WST-1 reagent was prepared from Roche Holding AG. Cytotoxicity assay kit II was prepared from the LDH bioscience source. All eukaryote cells were obtained from Pasteur Institute of Iran.

General procedures for the synthesis of compounds ***1–13***

To a solution of 0.1 g NaOH in 5 mL water, 1 mmol corresponding aldehyde was added under stirring. Then 1 mmol thiosemicarbazide was gradually added to the solution and under room temperature the mixture was stirred overnight. Then 5 mL ethyl alcohol was poured into the mixture, and refluxed for 1 h. After completion of the reaction, monitored by TLC using EtOAc/*n*-hexane (1:2) as eluent, the reaction mixture was filtered off to separate precipitate. Next, the precipitate was recrystallized from boiling ethanol to afford pure crystalline. The structure of novel compounds was confirmed by application of spectroscopic methods (The data are given in the Supplementary material to this paper).

Cell culture

Human breast cancer cells (MCF-7), human primary glioblastoma cells (U87), adenocarcinomic human alveolar basal epithelial cells (A549), human umbilical vein endothelial cells (HUVEC) and mouse embryonic fibroblast cell (3T3) as two normal cell lines were obtained from Pasteur Institute (Tehran, Iran) and incubated at 37 °C and 5 % CO_2 until flaunted the 75 % of the culture plate. Cells lines were grown in DMEM-F12 supplemented with 10 % FBS, 100 U mL^{-1} of penicillin-streptomycin.

Cytotoxicity studies

For cytotoxicity assay, a cell suspension containing 10^4 cells of each cell line were seeded into each well of a flat-bottomed 96-well plate and incubated for 24 h. On the following day, the medium was aspirated and 100 µl of each concentration (1000, 100, 10, 1, 0.1, 0.01, 0.001 ppm) of examined compounds were added into each well. Then plates were incubated 24 h at 37 °C and 5 % CO_2 atmosphere. On the next day 10 µl of WST-1 solution was added to each well and incubated for 4 h. Then the optical density (*OD*) was measured at 420 nm using an Elisa reader (BioTek Elx800). Cell viability was expressed as 100 % for untreated cells (control). All measures were performed in triplicates and the survival rate (%) was calculated as:[40]

$$\text{Survival rate} = 100(OD \text{ in treatment group}/OD \text{ in control group}) \quad (1)$$

The inhibitory concentration required for 50 % cytotoxicity (IC_{50}) was calculated using the Prism dose-response histogram (Graphpad Prism, Prism 9, version 9.3.0 (463) for Windows, 1992–2021 GraphPad Software, LLC), obtained by plotting the percentage of survival versus the concentration.

To further support the results obtained by WST-1 assay, the lactate dehydrogenase (LDH) release of U87, MCF-7 and 3T3 cells after 24 h incubation with compounds number 7, 9, 11 and 12 (as representative compounds) at 10, 100 and 1000 µg mL^{-1} concentrations were measured using the supernatant of the cell culture media by LDH cytotoxicity detection kit. A 10 µl of culture media on the cell was moved to a 96-well plate and 100 µl of LDH reaction mix was added to each well. The plate was then incubated at room temperature for 30–60 min when absorbance was read using the microplate reader. The absorbance was read at 492 nm using an automatic analyzer RA-1000 (Technicon, Ireland). Triton X (1 %) was used as positive control. The 650 nm reference absorbance was subtracted from the 450 nm absorbance reading to give the well absorbance. The cytotoxicity percentage was calculated as:

$$\text{Cytotoxicity} = 100[(A_{\text{sample}} - A_{\text{low control}})/(A_{\text{high control}} - A_{\text{low control}})] \quad (2)$$

where: low control was un-treated cells (control) and high control was treated cells with triton X 1 %.

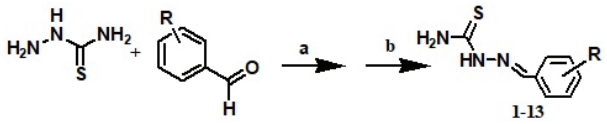
Statistical analysis

The data obtained from the WST-1 assay were evaluated by one-way ANOVA followed by Tukey's test in SPSS (statistical package for social sciences). Results were expressed as percentages of the control as the mean ± *SD*, and differences from the control groups were considered significant with $p < 0.05$. In addition, IC_{50} values of the substances were calculated by nonlinear regression analysis of triplicate experiments by the software Graphpad Prism.

RESULTS AND DISCUSSION

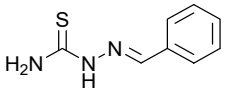
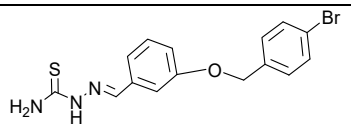
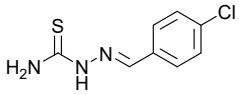
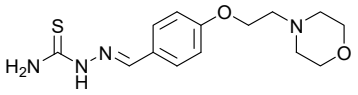
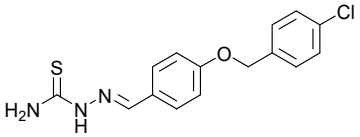
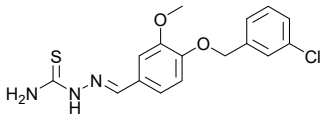
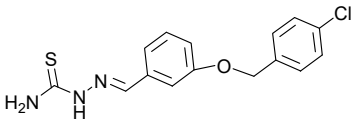
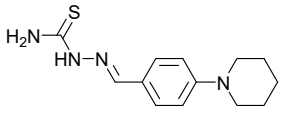
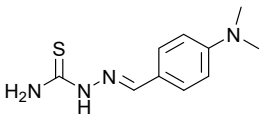
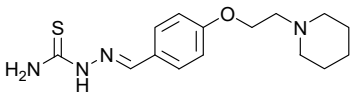
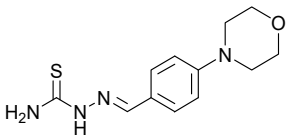
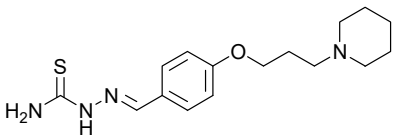
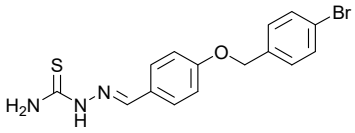
Chemistry

The target compounds were synthesized as outlined in Scheme 1. The condensation reaction between thiosemicarbazide and a variety of different substituted benzaldehyde or benzyloxybenzaldehyde compounds afforded the corresponding target benzylidene thiosemicarbazide derivatives (Table I). The identity of target compounds was confirmed by melting point, IR, ^{1}H- and ^{13}C-NMR spectra.



Scheme 1. Synthetic route for the target compounds. Reagents and conditions: a) NaOH solution, rt, overnight; b) EtOH, reflux, 1 h.

TABLE I. The structure of the synthesized compounds

Cmpd. No.	Structure	Cmpd. No.	Structure
1		**8**	
2		**9**	
3		**10**	
4		**11**	
5		**12**	
6		**13**	
7			

Cytotoxicity assay

The cytotoxic effect of thiosemicarbazide derivatives (13 compounds) on MCF-7, U87, A549, HUVEC and 3T3 cell lines was determined by WST-1 and LDH assays. The toxicity of the synthesized compounds was investigated in the concentration range of 0.001 to 1000 mg L^{-1}. However, to be in line with the published results on the medicinal chemistry, the concentration of compounds in

micromolar ranged from the least 2.75×10^{-3} (cmpd. **7**) to 5590 μM (Cmpd. **1**) which is shown in Table II. The WST-1 results are depicted as dose-response histograms in Figs. 2 and 3 and IC_{50} values in Table III. The dose-response histograms shown in Figs. 2 and 3 indicate that MCF-7 and HUVEC cell lines are the most sensitive ones against all tested compounds. Also, 3T3 and U87 cell lines were considered as the most resistant ones against all compounds at concentrations ≤ 10 μg mL^{-1}. In addition, compounds **5** with IC_{50} = 7.3 μM against MCF-7, **7** with IC_{50} = 24.73 μM against U-87, and **11** with IC_{50} of 46.37 and 46.60 μM against A549 and MCF-7, respectively, accounted for compounds with the most effective toxicity profiles. Based on statistical analysis, the results showed that the compounds used in the concentration ranges 0.001–1000 mg L^{-1} tested on MCF-7, A549 and HUVEC cell lines exhibited a significant difference ($p < 0.05$).

TABLE II. The concentration of studied compounds applied in biological assays and IC_{50} in terms of μM

Cmpd. No.	c / mg L^{-1}						
	0.001	0.01	0.1	1	10	100	1000
1	5.59×10^{-3}	5.59×10^{-2}	5.59×10^{-1}	5.59	55.9	559	5590
2	4.68×10^{-3}	4.68×10^{-2}	4.68×10^{-1}	4.68	46.8	468	4680
3	3.13×10^{-3}	3.13×10^{-2}	3.13×10^{-1}	3.13	31.3	313	3130
4	3.13×10^{-3}	3.13×10^{-2}	3.13×10^{-1}	3.13	31.3	313	3130
5	4.50×10^{-3}	4.50×10^{-2}	4.50×10^{-1}	4.50	45	450	4500
6	3.79×10^{-3}	3.79×10^{-2}	3.79×10^{-1}	3.79	37.9	379	3790
7	2.75×10^{-3}	2.75×10^{-2}	2.75×10^{-1}	2.75	27.5	275	2750
8	2.75×10^{-3}	2.75×10^{-2}	2.75×10^{-1}	2.75	27.5	275	2750
9	3.25×10^{-3}	3.25×10^{-2}	3.25×10^{-1}	3.25	32.5	325	3250
10	2.86×10^{-3}	2.86×10^{-2}	2.86×10^{-1}	2.86	28.6	286	2860
11	3.82×10^{-3}	3.82×10^{-2}	3.82×10^{-1}	3.82	38.2	382	3820
12	3.27×10^{-3}	3.27×10^{-2}	3.27×10^{-1}	3.27	32.7	327	3270
13	3.12×10^{-3}	3.12×10^{-2}	3.12×10^{-1}	3.12	31.2	312	3120

It is known that the free electrons on elements such as N, S, and O have a high tendency to bind to metal ions such as iron and copper. Because cells need metallic elements such as iron, zinc, and magnesium to grow, especially in the G/S1 phase of cell proliferation, so iron deficiency stops cell proliferation and growth.[41]

Cancer cells tend to multiply rapidly thus iron deficiency can affect them much more. Therefore, iron chelators can have anti-tumor effects.[42]

Metal chelators have the potential to inhibit iron uptake from iron-carrying proteins, inhibit the expression of iron-containing enzymes such as ribonucleotide reductase, and inhibit the formation of redox-active iron complexes that produce reactive oxygen species.[41,43] In this project, MCF-7, among cancer cell lines,

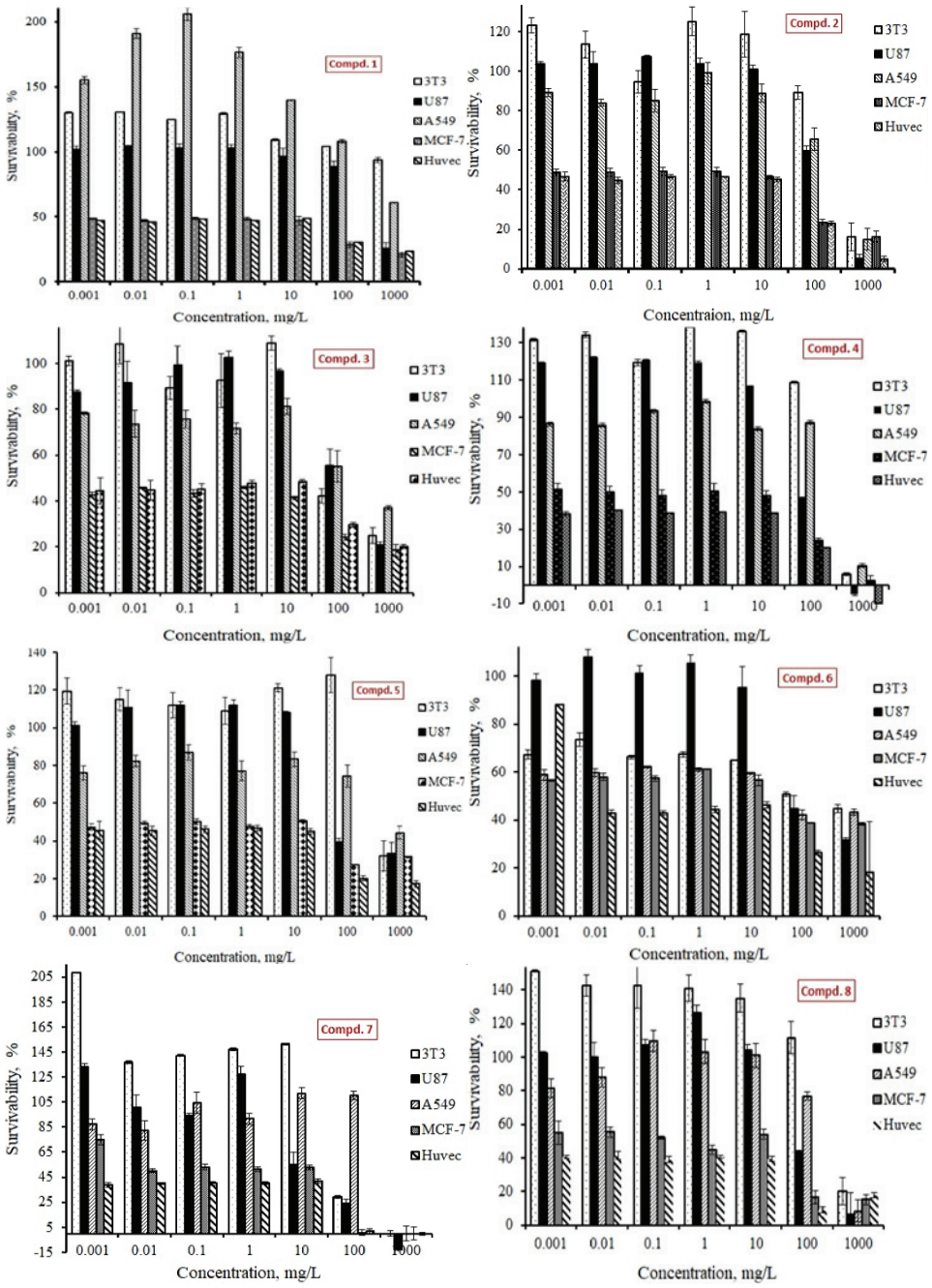


Fig. 2. The dose-response histogram of compounds 1-8 on MCF-7, U87, A549, HUVEC and 3T3 cell lines determined by WST-1 assay.

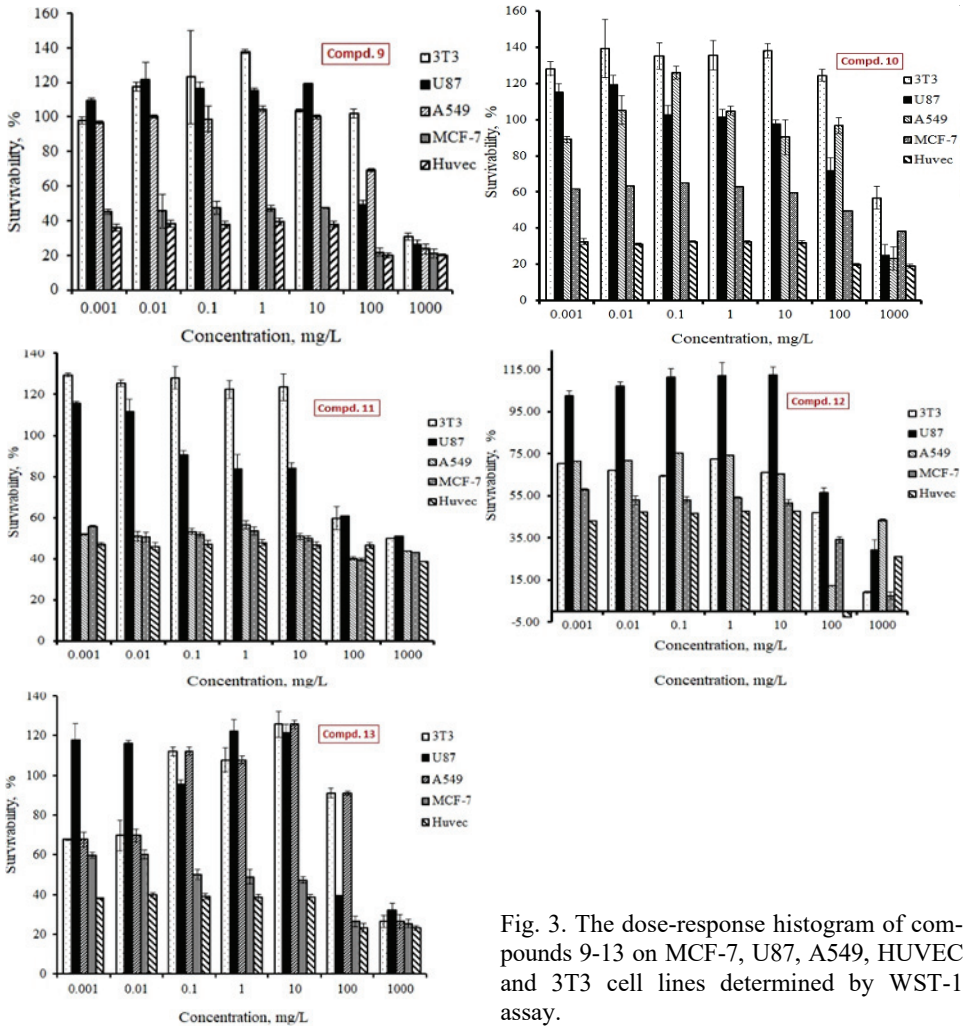


Fig. 3. The dose-response histogram of compounds 9-13 on MCF-7, U87, A549, HUVEC and 3T3 cell lines determined by WST-1 assay.

was the most sensitive cell line against all concentrations of synthesized compounds. In contrast, U87 cell line was resistant to almost all compounds at concentrations below 100 ppm and showed cell survival of more than 80 %. On the other hand, healthy cell lines showed completely different behaviour. For example, the cell line 3T3, which originates from mouse fibroblasts, showed resistance to all compounds at all concentrations, while HUVEC cell line, originating from the human umbilical cord, appeared to be sensitive to all compounds, even at low concentrations, this cell line did not show high cell survival. This sensitivity and resistance phenomena may be due to the cellular origin and the type of tissue from which the cells were extracted.

TABLE III. The IC_{50} values of studied compounds

Cmpd. No.		A549	MCF-7	U-87	3T3	HUVEC
1	IC_{50} / μM	344.53	330.34	1509.44	67.04	506.82
	IC_{50} / mg L^{-1}	61.67	59.13	270.19	12	90.72
2	IC_{50} / μM	689.46	218.97	602.81	569.09	414.75
	IC_{50} / mg L^{-1}	147.20	46.75	128.70	121.50	88.55
3	IC_{50} / μM	305.76	133.05	305.10	265.73	290.89
	IC_{50} / mg L^{-1}	97.69	42.51	97.48	84.90	92.94
4	IC_{50} / μM	1114.65	283.41	265.20	416.59	396.56
	IC_{50} / mg L^{-1}	356.13	90.55	84.73	133.10	126.70
5	IC_{50} / μM	598.65	7.30	201.13	3862.61	180.45
	IC_{50} / mg L^{-1}	132.90	1.62	44.65	857.50	40.06
6	IC_{50} / μM	53.18	84.55	147.05	158.41	339.51
	IC_{50} / mg L^{-1}	14.04	22.32	38.82	41.82	89.63
7	IC_{50} / μM	1929.12	62.75	24.73	128.30	188.76
	IC_{50} / mg L^{-1}	702.20	22.84	9.00	46.70	68.71
8	IC_{50} / μM	346.43	175.25	200.00	350.14	52.80
	IC_{50} / mg L^{-1}	126.10	63.79	72.80	127.45	19.22
9	IC_{50} / μM	380.52	216.69	275.91	513.47	51.30
	IC_{50} / mg L^{-1}	117.20	66.74	84.98	158.15	15.80
10	IC_{50} / μM	603.95	317.31	383.69	410.30	186.29
	IC_{50} / mg L^{-1}	211.08	110.90	134.10	143.40	65.11
11	IC_{50} / μM	46.37	46.60	762.21	174.31	907.79
	IC_{50} / mg L^{-1}	12.15	12.21	199.70	45.67	237.84
12	IC_{50} / μM	350.65	540.20	296.05	354.58	754.38
	IC_{50} / mg L^{-1}	107.30	165.30	90.59	108.50	230.84
13	IC_{50} / μM	217.13	108.94	230.25	827.69	102.78
	IC_{50} / mg L^{-1}	69.48	34.86	73.68	264.86	32.89

The study carried out by Potůčková and co-workers has shown that the compound 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone reveals high toxicity effects on HL-60 (human leukemia), MCF-7 and HCT116 (human colon cancer) and moderate toxicity on A549 and healthy cell line H9C2 (neonatal mouse heart cell). They claimed that 3T3 fibroblasts were the most resistant of all the cell-types to every agent examined.[44] In the research by Serda and co-workers[28] on thiosemicarbazide skeletons, derivatives that exhibited good activity as anti-cancer agents were introduced, among those the two compounds, depicted in Fig. 4, displayed the greatest promise. They attributed the anti-cancer ability of their compounds to the complexing power of thiosemicarbazides. Their study on the structure-activity relationship showed that the combination of donor atoms N, N, S in thiosemicarbazone backbone had a crucial role in the anti-cancer activity of their compounds, rather than the existence of different fragments in R position. Also, thiosemicarbazones appear to elevate the level of reactive oxygen species that disrupt the antioxidant activity of mitochondria triggering apoptosis pro-

cess.[45] The presence of aromatic rings, attached to thiosemicarbazone moiety, increase the anti-cancer activity by DNA intercalating ability.[44]

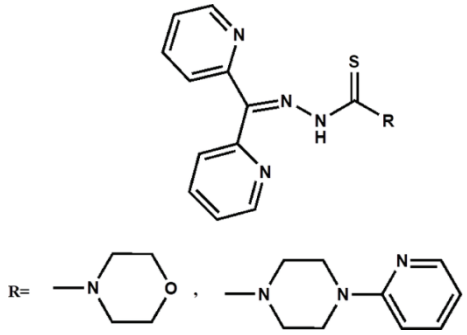


Fig. 4. Two thiosemicarbazones with good antiproliferative activity from Serda and co-workers' work.[28]

The cytotoxicity determinations were performed to confirm the toxycity effects of the synthesized compounds by WST-1 and LDH assays. Fig. 5 shows the cell viability (WST-1 assay) and cell mortality (LDH assay) results of three cell lines (MCF-7, U87 and 3T3) after treatment with four representative compounds (No. **7**, **9**, **11** and **12**) at 10, 100 and 1000 μg mL^{-1} concentrations.

First of all, WST-1 assay was done at different concentrations to recognize the IC_{50} values. All of the examined compounds showed a quality of being toxic to cells. The WST-1 technique is a common colorimetric approach to assess cytotoxicity or cell viability through determination of mitochondrial function of cell by measuring activity of mitochondrial enzymes. Also, to confirm the cell membrane damaging effects of the synthesized materials, the LDH enzyme activity was measured on the cells after being treated with three of the highest concentrations used in this project, which showed toxicity to all the cells.

Considering our WST-1 results that 3T3 and U87 cells below concentrations less than 10 μg mL^{-1} were evaluated as the most resistant cells, hence for LDH assay these cells were used in concentrations above 10. From the other cells that appeared as sensitive cells in this project, MCF-7 cell was also selected for LDH assay. Also, since the synthesizad compounds exerted high toxicity on most of the cells at high concentrations, only three high concentrations were used for LDH assay. Among all 13 synthesized compounds, only 4 representative ones were used that had good toxicity profiles on the three selected cell lines.

According to the results, all of the examined compounds showed some disturbing effect on the cell membrane and secreted more LDH enzyme. The LDH assay is used for the assessment of cytotoxicity mediated by toxic compounds and the LDH enzyme release from damaged cells. In this method, if the cells are attacked aggressively, due to the membrane destruction and cell damage, there would be more enzyme level secretion and the subsequent mortality is reported to be higher.[46] However, if cells are attacked by cellular mechanisms, low enzyme release is reported due to the lack of cell damage.

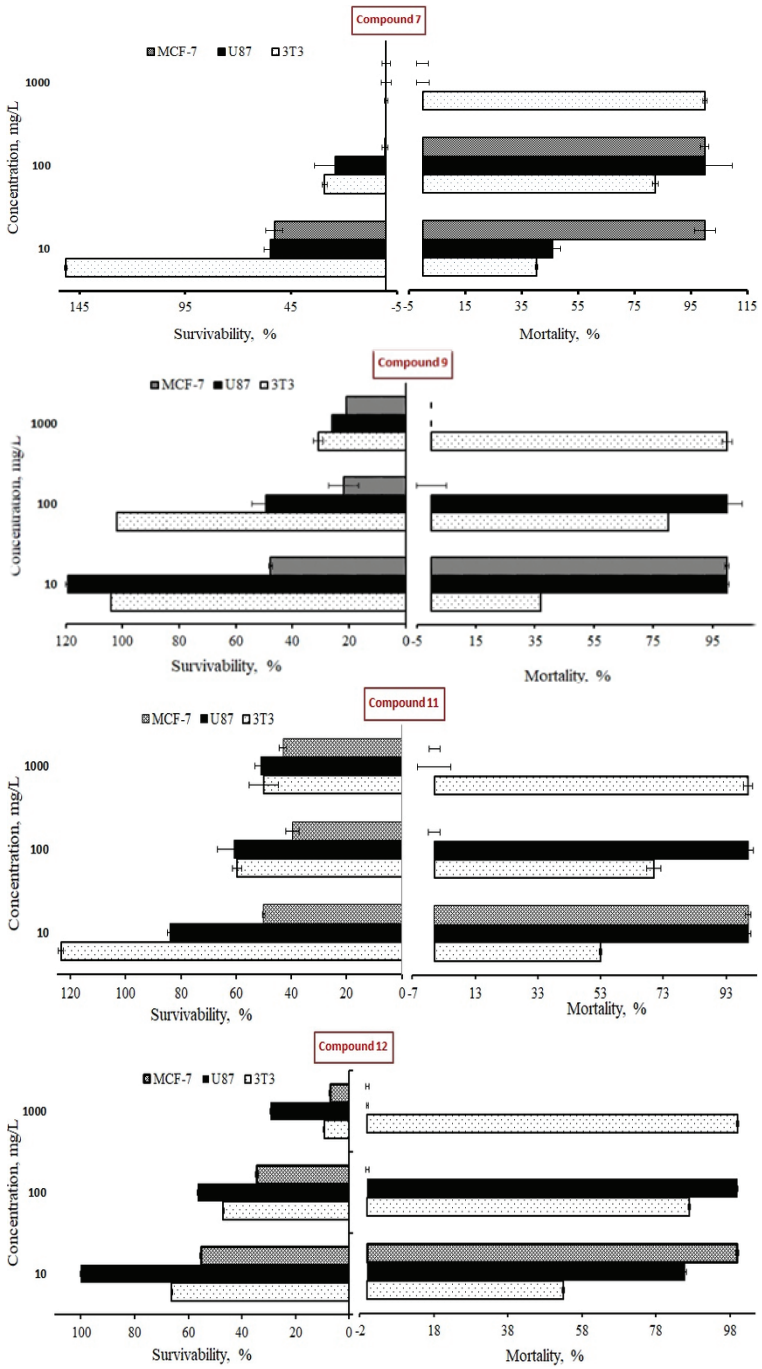


Fig. 5. The MCF-7, U87 and 3T3 cells viability (WST-1) and mortality (LDH) after treatment with various concentrations of compounds No. **7**, **9**, **11** and **12**.

The results shown in Fig. 5 indicate that the mechanism of action of cytotoxicity of benzylidene thiosemicarbazides on cells can be addressed differently.

Against 3T3 cells, the studied compounds show their toxicity by the mechanism of cell damage and cell destruction. In a way, the more toxic the compound, the lower the cell survival rate, the higher the LDH release, which results in a higher mortality rate because cell death is achieved through cell destruction.

At high concentration, *i.e.*, 1000 mg L^{-1}, the studied compounds displayed their toxicity against U87 and MCF-7 cell lines through intracellular mechanisms and not through cell membrane damage, so that despite the low cell survival rate at this concentration, the level of LDH secretion is at its lowest level. These intracellular mechanisms can be attributed as though specified complexes probably induce apoptotic death. Compounds **9**, **11** and **12** at concentration of 100 mg L^{-1} also induced MCF-7 cell death by intracellular mechanism, but at low concentration of 10, all four benzylidene thiosemicarbazide derivatives appear to induce cell destruction leading to the death of U87 and MCF-7 cells, and consequently the rate of LDH release is proportional with the rate of cell death.

Based on Fig. 5, the behavior of U87 cell after treatment with compounds **9**, **11** and **12** at a concentration of 10 mg L^{-1} is strange. Whether this can be addressed as false positive WST-1 result – due to variable metabolic behavior under different cell culture conditions,[54] or impermeability of WST-1 and its reduction reaction at the cell surface or at the level of the plasma membrane *via* trans-plasma membrane electron transport[55] – or whether this is attributed to high LDH release – due to high spontaneous release caused by poor condition of the cells used,[56] or variability of LDH activity within different cell types[57] or other conflicting parameters are involved , this remains open for further investigations.

*Selectivity index (*SI*)*

The selectivity index (*SI*) is a measure of how differentiated a compound is against cancer cells compared to healthy cells. To evaluate the toxicity of the studied compounds, the equation 2 was used to calculate the selectivity index:[47]

$$SI = \frac{IC_{50}(\text{healty cell})}{IC_{50}(\text{cancer cell})} \quad (2)$$

The results of the selectivity index calculation are shown in Table IV. Theoretically the higher the *SI*, the safer a compound would be during treatment for a given infection and the higher the potential to be developed as a safe drug.[48] According to the acceptance criteria for selectivity index, selective compounds against cancer cells should have $SI > 10$; and compounds with *SI* lower than 10 but higher than 1 could be considered as non-selective.[47] A lower *SI* value (≥ 3) has been proposed by Weerapreeyakul *et al.* for classifying a promising anti-cancer agents.[49] However Angel Quispe *et al.* a further lower *SI* value (> 1) and rec-

ommended for substances to be more cytotoxic to tumor cells than to normal cells.[50] Vonthron-Sénécheau *et al.* suggested an acceptance criterion of $SI \geq 10$ for plant extracts with promising antimalarial activity.[51] Compounds with $SI < 2$ are not considered as good candidates for antimalarial activity.[48] However, recently Famuyide *et al*. described $SI > 1$ for plant extracts with antibacterial effect as bioactive and non-toxic agents.[52] Due to many variations that have been presented about the selectivity acceptance criteria in different journals as mentioned above, compounds with SI below 1 are not recommended for further studies.

TABLE IV. The selectivity index values based on 3T3 or HUVEC as healthy cells

Cmpd. No.	Based on 3T3			Based on HUVEC			
	A549	MCF-7	U-87	A549	MCF-7	U-87	3T3
1	0.19	0.20	0.04	1.47	1.53	0.34	0.13
2	0.83	2.60	0.94	0.60	1.89	0.69	1.37
3	0.87	2.00	0.87	0.95	2.19	0.95	0.91
4	0.37	1.47	1.57	0.36	1.40	1.50	1.05
5	6.45	529.32	19.20	0.30	24.73	0.90	21.41
6	2.98	1.87	1.08	6.38	4.02	2.31	0.47
7	0.07	2.04	5.19	0.10	3.01	7.63	0.68
8	1.01	2.00	1.75	0.15	0.30	0.26	6.63
9	1.35	2.37	1.86	0.13	0.24	0.19	10.01
10	0.68	1.29	1.07	0.31	0.59	0.49	2.20
11	3.76	3.74	0.23	19.58	19.48	1.19	0.19
12	1.01	0.66	1.20	2.15	1.40	2.55	0.47
13	3.81	7.60	3.59	0.47	0.94	0.45	8.05

In the study of the selectivity index of the studied compounds in relation to HUVEC as healthy cell, these compounds showed the best selectivity characterrization against MCF-7 cancer cell line. Against MCF-7, the *SI* of compounds **5** and **11** far exceeded the value of 10 (more cytotoxic for tumor cells), unlike compound **9**, with the least *SI* value, which only reached a value of 0.24 (more cytotoxic for normal HUVEC cell). Also, compound **11** displayed a $SI = 19.58$, as the most promising compound against A549 and at the same time compound **7** with $SI = 7.63$ accounted for the most selective one against U-87 when compared with HUVEC. Also compounds **6** and **11** exerted a $SI > 2$ and > 1, respectively, against all three cell lines, when selectivity ratio was measured based on HUVEC cytotoxicity.

Instead, all compounds had selective toxicity against cancer cells in relation to 3T3 cells. According to this, all compounds except **1** and **12**, presented $SI > 1$ against MCF-7. Compounds **5** and **13** exerted a relatively good safe margin and promising anti-MCF-7 activity; compounds **5** and **7** against U-87 and compounds **5**, **13** and **11** against A549 showed the same selectivity profile when their cytotoxicity was compared with 3T3 cytotoxicity. In general, compound **5** presented

the least cytotoxicity against all three cancer cell lines and the highest cytotoxicity against 3T3. In the next ranks, compounds **13**, **9** and **6** had respectively acceptable toxicity ($SI > 1$) against all three cancer cell lines compared to 3T3. Based on 3T3, compound **7** was considered as the most cytotoxic agent against A549 ($SI = 0.07$); compound **1** against MCF-7 with $SI = 0.20$ and compound **11** against U-87 with $SI = 0.23$ were considered toxic, too.

Generally, compounds **6** and **11** among other compounds showed the best selectivity against all three cancer cells. This is while compounds **6** and **11** displayed relatively low IC_{50} against all three cancer cells.

By determining the selectivity of these compounds against HUVEC,[53] the effect of the synthesized compounds on angiogenesis can be carefully estimated. By measuring the selectivity index of the compounds against HUVEC in relation to 3T3 cell, a cautious estimation of their anti-angiogenic potential can be apprized, although specific test for anti-angiogenesis evaluation should be performed to speak certainty in this regard.

Looking at the last column in the selectivity indices in Table IV, it can be said that compounds **5** and **9** (*SI* of 21.41 and 10.01, respectively) can be strongly considered as anti-angiogenic agents. Compounds **13** and **8** (*SI* of 8.05 and 6.63, respectively) are in the second place and compounds **10**, **2** and **4** with respective *SI* of 2.2, 1.37 and 1.05 ranked the third place. The other compounds can be considered as general toxic.

CONCLUSION

In this study, a series of benzylidene thiosemicarbazide derivatives were synthesized and tested for their cytotoxicity against three different cancer cell lines as well as two different normal cell lines. Although most of the derivatives possessed weak cytotoxic activity against U-87 and, to somehow, A549 cell lines, they exhibited good activity against MCF-7. Among healthy cell lines the 3T3 cell line showed resistance to all compounds at all concentrations, while HUVEC cell line appeared to be sensitive to all compounds, even at low concentrations. Based on this study, compound **5** was found to be the most potent cytotoxic agent against MCF-7 and compounds B and B against A549 and MCF-7 showed the highest cytotoxicity. This is while compounds **6** and **11** displayed the best selectivity profiles. Based on LDH results, we can say that benzylidene thiosemicarbazides show toxicity through different actions. At high concentrations they show their toxicity by possible formation of specified complexes inducing apoptotic mechanisms whist at low concentration, they follow destructive approaches.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/10908, or from the corresponding author on request.

ИЗВОД

СИНТЕЗА НЕКИХ ДЕРИВАТА БЕНЗИЛИДЕН-ТИОСЕМИКАРБАЗИДА И ПРОЦЕНА ЊИХОВЕ ЦИТОТОКСИЧНОСТИ НА U87, MCF-7, A549, 3T3 И HUVEC ЋЕЛИЈСКИМ ЛИНИЈАМА

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Хомеостаза гвожђа се мења у туморима као одговор на поремећај у експресији протеина зависних од гвожђа. Због тога, хелатори гвожђа чине ћелије рака рањивијим на недостатак гвожђа. Једињења која имају тиосемикарбазидну структуру са способношћу формирања металних комплекса имају потенцијал да делују антиканцерно. Серија деривата тиосемикарбазида је дизајнирана и успешно синтетизована, а њихова цитотоксичност је затим тестирана на неким канцерогеним, као и на лабораторијским нормалним моделним системима, коришћењем колориметријског теста заснованог на WST-1 реагенсу. Према резултатима цитотоксичности, нека једињења су показала висок токсични ефекат и на канцерогене и на здраве ћелијске линије. Резултати испитивања токсичности на ћелијским линијама U87 и A549 су показали преживљавање мање од 50 % при свим концентрацијама већим од 10 ppm за сва синтетизована једињења. Ћелијска линија MCF-7 је показала приближно исто понашање и имала је преживљавање мање од 60 %. 3T3 у поређењу са HUVEC ћелијском линијом показао је потпуно другачије понашање у односу на синтетизована једињења и имао је преживљавање више од 50 %. Измерен је и индекс селективности и на основу резултата студије може се закључити да профил цитотоксичности синтетизованих једињења на 3T3 ћелијској линији показује значајну разлику, што указује на добар антиканцерогени ефекат ових једињења.

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