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EVALUATION OF BIOLOGICAL PROPERTIES OF SOME NITROBENZOFURAZAN DERIVATIVES CONTAINING A SULFIDE GROUP**

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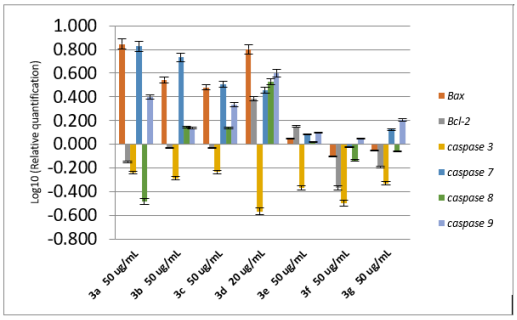
The purpose of our study is to evaluate the antitumor activity of some previously reported nitrobenzofurazan derivatives. In this regard, their cytotoxic effect, influence on eukaryotic cell cycle, and on some genes implicated in apoptosis were evaluated. Compounds **3b**, **3c** and **3d** induced apoptosis through both intrinsic and extrinsic pathways, while compound **3a** activated only the intrinsic pathway. The most toxic derivative proved to be compound **3d**; this compound also slightly increased the S phase and decreased the G0/G1 phase of the cell cycle in a dose dependent manner. Hydrophobicity (log P), polar surface area (PSA) and molecular volumes were calculated and correlated with some biological properties.



INTRODUCTION

Cancer is a group of diseases that involves the abnormal growth of cells resulting from an imbalance between proliferation and apoptosis, the programmed cell death. Apoptosis involves the activation of caspases whose protease activity is essential for apoptosis and can be initiated either *via* cell-surface death-receptors, the so-called death receptor (or extrinsic pathway), or through the mitochondrial, intrinsic pathway of apoptosis.[1,2] The most commonly deregulated form of cell death in cancer is the mitochondrial pathway of apoptosis. The Bcl-2 family controls cell death primarily by direct binding interactions and regulates the commitment to apoptosis by controlling permeabilization of the mitochondrial outer membrane.[3] Bcl-2 family members are located in the outer membrane of the mitochondria and function by blocking the release of cytochrome C from the mitochondria. In contrast to Bcl-2 family members, the insertion of Bax family members into the mitochondrial membrane induces the release of cytochrome C and the induction of apoptotic cell death.[4] Cell death plays a major role in cancer treatments, serving as the main effector function of many anti-cancer therapies. Various compounds with potential anti-cancer activity that induce apoptosis are reported in the literature. For example, a dual-targeted cascade-responsive multifunctional polymer micelle with hyaluronidase/redox/light multilevel responses was developed in order to

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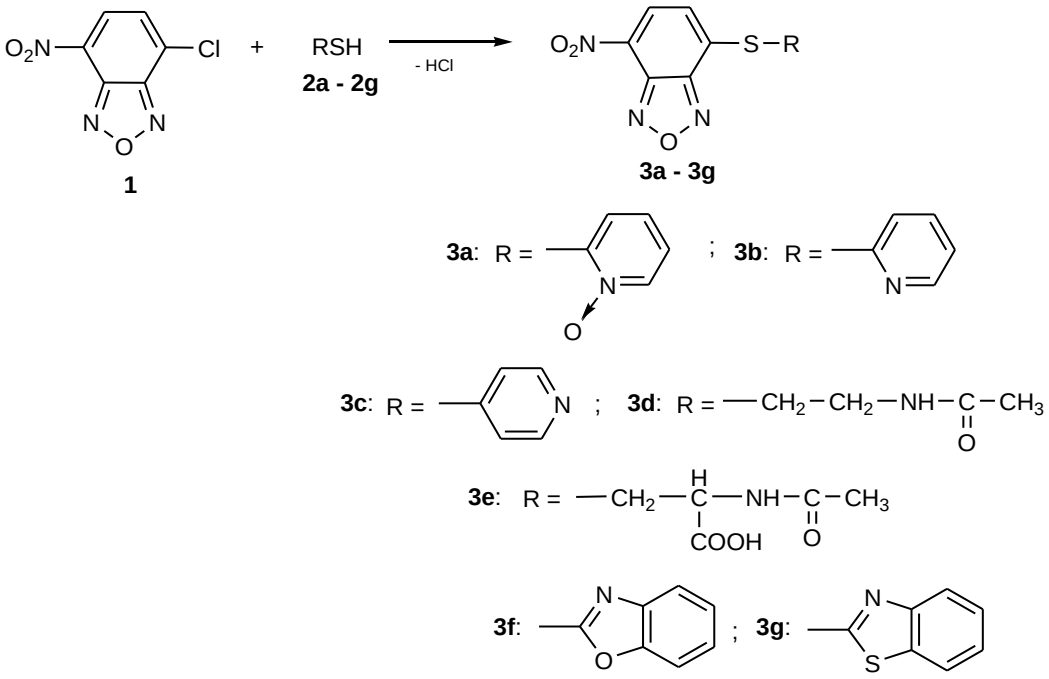
induce mitochondrial apoptosis in cancer therapy. This nano-formulation shows highly effective anticancer efficacy *in vivo*.[5] Additionally, the selective toxicity of a synthesized chalcone-ferrocenyl derivative as a selective cycloxygenase-2 inhibitor in pediatric acute lymphoblastic leukemia (ALL) and healthy B-lymphocytes, as well as isolated mitochondria obtained from them, was investigated. This compound can selectively induce cellular and mitochondrial toxicity in cancerous ALL B-lymphocytes.

The nitrobenzofurazan derivatives have been previously reported to possess broad applications in medicinal chemistry and biology, such as anti-protozoa, antibacterial, calcium channel modulating properties,[6-9] antifungal agents against phytopathogenic fungi,[10] antiviral drugs, inhibitors of influenza A virus,[11,12] antirheumatic drugs,[13] ligands of the polyamine transport system of *Leishmaniadonovani*,[14] anticancer drugs, fluorescent probes for bioimaging biothiols,[15] and for the determination of cysteine in living cells.[16]

In this study we explored the cytotoxic activity of seven thioethers **3a**–**3g** (Scheme 1) having a nitrobenzofurazan moiety attached *via* the sulfur atom ("pivot atom") as potential antitumor drugs. We reported in two previous papers about five NBD-thioethers (**3a**–**3e**) prepared by an S_NAr process from the electrophile NBD-Cl (4-chloro-7-nitrobenzo furazan), (**1)** and thiolic nucleophiles **2a**–**2e** (RSH, Scheme 1),[17] where: R = 2-pyridinethiol-1–oxide (**2a**), 2-pyridinethiol (**2b**), 4-pyridinethiol (**2c**), *N*-acetylcysteamine (**2d**), *N*-acetylcysteine (**2e**), in ethanol with sodium hydrogen carbonate (Scheme 1), and two NBD thio-ethers (**3f, 3g**) prepared from NBD-Cl, (**1)** with 2-mercaptobenzoxazole and 2-mercaptobenzothiazole, respectively.[18] The compounds **3a** (2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio] pyridine-1-oxide) and **3b** (2-(2-[7-nitrobenzo[c] [2,1,5]oxadiazol-4-yl))thio]pyridine were identified as inhibitors of KIX-KID interaction from screening the NCl-diversity set of compounds using a split renilla luciferase assay.[19] The compound **3a** enhance tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) and triggers downstream signaling pathways and other receptor tyrosine kinases in cancer cells.[20] Also, the compound **3d** was evaluated for its cytotoxic effect on the human osteosarcoma U2OS cell line.[21]



Scheme 1 – Synthesis of thioethers **3a**–**3g**.[17,18]

RESULTS AND DISCUSSION

The cytotoxic effect of tested compounds **3a**–**3g**, on eukaryotic cells was evaluated using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. In this regard, the tested compounds were quantitatively assayed on HCT-8 cells for 24 hours using serial binary dilutions ranging from 500 μg/mL to 0.24 μg/mL (Fig. 1). The most toxic compound proved to be **3d** (IC_{50} = 7.76 μg/mL), followed by **3a** (IC_{50} = 29.97 μg/mL), **3c** (IC_{50} = 57.50 μg/mL) and **3b** (IC_{50} =

60.66 μg/mL), while compounds **3f** (IC_{50} = =137.7μg/mL) and **3g** (IC_{50} = 120.4μg/mL) were less toxic. The compound **3e** did not affect the viability and metabolic activity of HCT-8 cells. For further analyses we selected a representative concentration for all compounds (50 μg/mL) that will affect the cells without causing cell death.

The influence of the NBD thioethers **3a–3g** on the cell cycle was determined on HCT-8 cells. In this regard, human colon cancer HCT-8 cells were treated for 72 hours with 50 μg/mL of the tested compounds (Fig. 2). The compound **3b** determined a slight increase of the G0/G1 phase and a decrease of the G2/M phase.

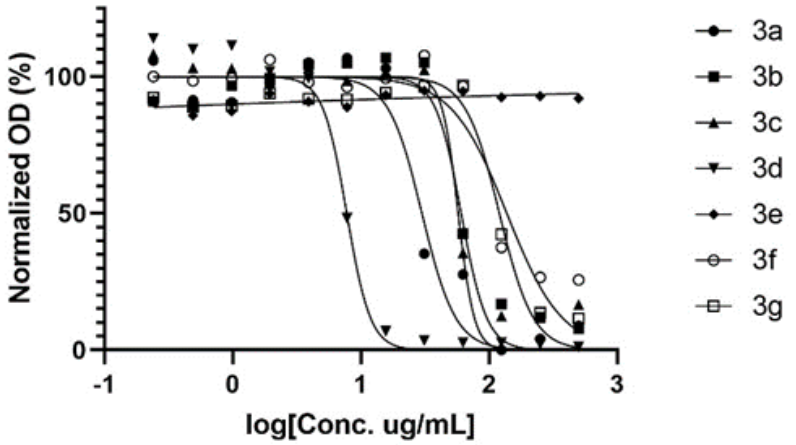
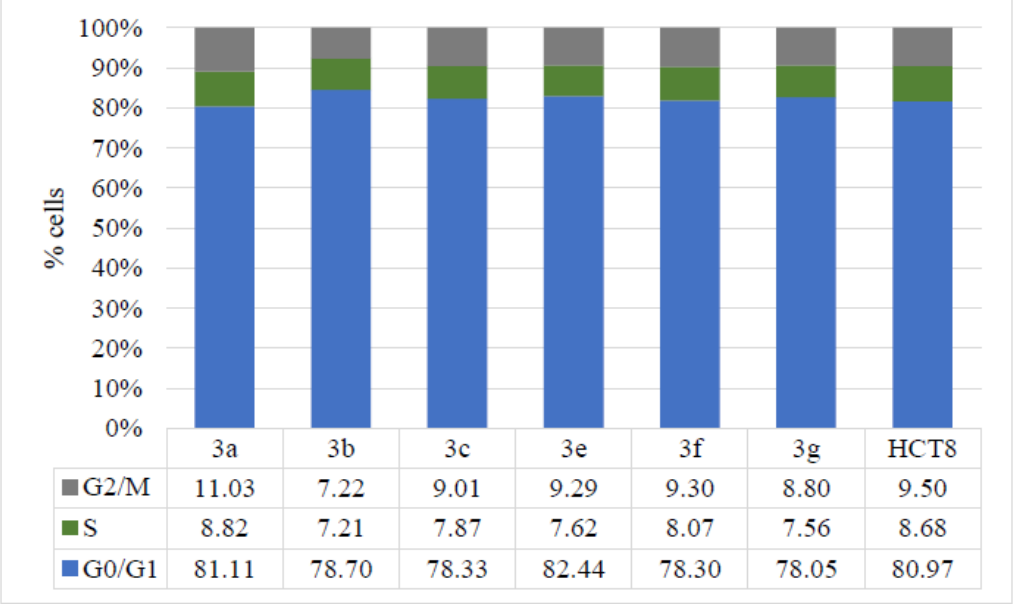


Fig. 1 – The cytotoxic effect of tested compounds. HCT8 cells were treated with tested compounds for 24 hours at different concentrations. The cytotoxicity was evaluated by the MTS assay and IC_{50} values were calculated using GraphPad Prism software.



	3a	3b	3c	3e	3f	3g	HCT8
G2/M	11.03	7.22	9.01	9.29	9.30	8.80	9.50
S	8.82	7.21	7.87	7.62	8.07	7.56	8.68
G0/G1	81.11	78.70	78.33	82.44	78.30	78.05	80.97

Fig. 2– The influence of the tested compounds on the cell cycle in eukaryotic cells. HCT8 cells were treated with 50 μg/mL of the tested compounds for 72 hours and cell cycle distribution was analyzed by flow cytometry using propidium iodide staining. The cell cycle phase distribution was shown by bar diagram.

The compound **3d** proved to be the most toxic, inducing cell death at a tested concentration of 50 μg/mL. To elucidate the mechanism of action, this compound was tested for 72 hours at lower concentrations: 20 μg/mL, 10 μg/mL, 5 μg/mL, and 1 μg/mL (Fig. 3). Treatments with 20 μg/mL, 10 μg/mL, and 5 μg/mL of **3d** induced cell death in HCT-8 cells, as evidenced by the presence of sub-G0/G1 (left) peaks. Additionally, compound **3d** slightly increased the S phase and decreased the G0/G1 phase in a dose dependent manner, associated with the induction of cell death through apoptosis/necrosis.

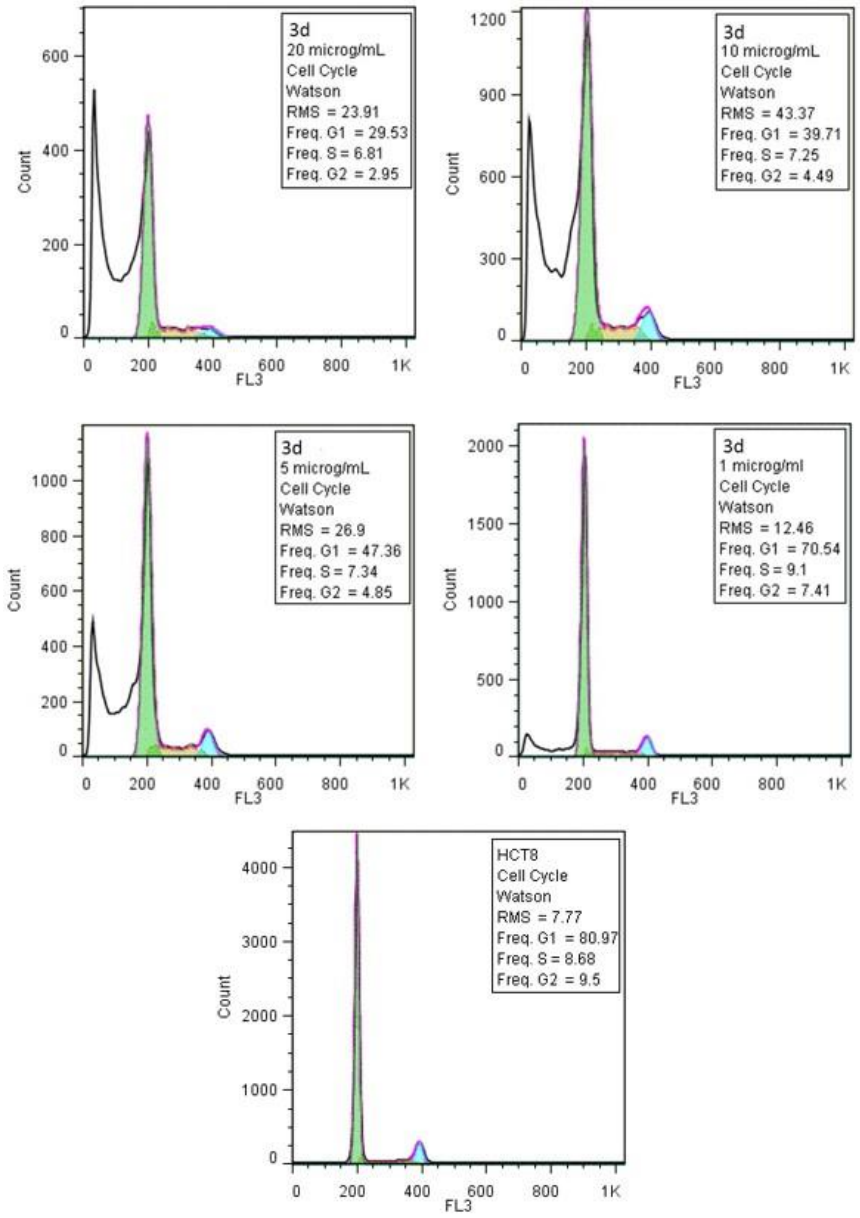


Fig. 3 – The influence of compound **3d** on the cell cycle in eukaryotic cells. HCT8 cells were treated with compound **3d** at different concentrations for 72 hours and cell cycle distribution was analyzed by flow cytometry using propidium iodide staining. The histograms represent the number of cells per channel (vertical axis) versus DNA content (horizontal axis). The distribution of cell phases is represented on the untreated HCT-8 cells (control) histogram.

The cytotoxic effect of tested compounds **3a**–**3g** was also evaluated at the molecular level by analyzing the expression levels of some genes implicated in apoptosis through real time PCR (Figure 4). Compounds **3a**, **3b**, **3c** and **3d** increased the expression levels of activating *caspase 9* and effector *caspase 7*. It appears that these compounds induced apoptosis through the mitochondrial stress pathway, characterized by the release of cytochrome C from mitochondria and its interaction with Apaf-1, leading to the activation of caspase 9.[22] The increased gene expression of *caspase 7* can be correlated with cells detachment observed using microscopy after a 24 hour treatment, as one of the functions of this caspase is to detach cells from the extracellular matrix (ECM).[22] The increased expression level of *caspase-8* induced by compounds **3b**, **3c** and **3d** indicates a possibility of apoptosis activation, also, via extrinsic pathway. Compounds **3a**, **3b**, **3c** and **3d** also increased *Bax* gene expression level, while *Bcl-2* gene expression was decreased by **3b**, **3c**, **3d**, and slightly increased by **3d**. Nonetheless, the *Bax/Bcl-2* ratio was positive indicating the induction of apoptotic program.

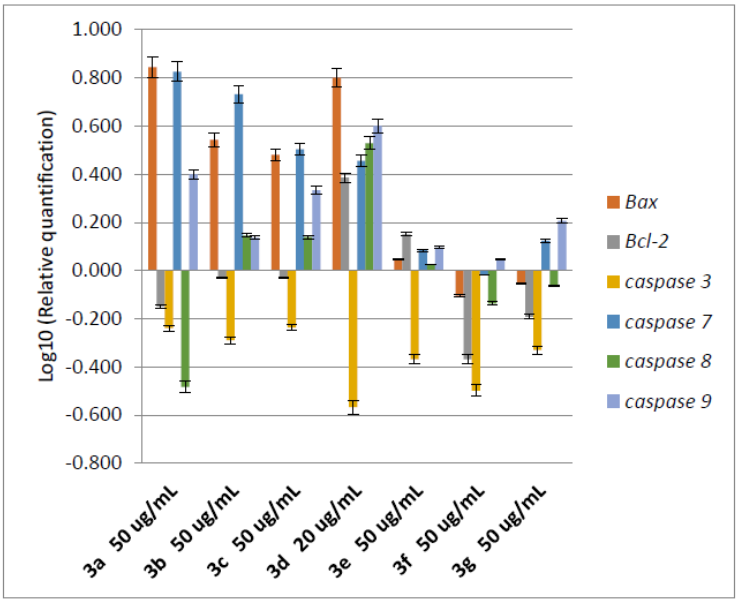


Fig. 4 – The influence of the tested compounds on the expression of some genes implicated in apoptosis induction. HCT8 cells were treated with the tested compounds for 72 hours and gene expression was analyzed by real-time PCR. mRNA expression was normalized to that of GAPDH and then standardized to that in untreated cells (control).

To gather more information about bioactivity of our compounds, some molecular calculations were performed using MolInspiration software.[23] Thus, hydrophobicity (lipophilicity) denoted as log P, polar surface area (PSA) and molecular volume (V) were calculated and compiled in Table 1. Biological interactions play an important role in medicinal sciences. Among molecular properties log P is important because the biological activity is correlated in quantitative structure activity relationships with the compound's capacity to cross cell membrane. Bioavailability, drug absorption, drug–receptor interaction and toxicity are corelated with the polarity and volume of the molecule.

Table 1

Calculated values of log P, PSA and V for specified compounds

Compound	Log P	PSA (A^2)	V (A^3)
3a	1.58	110.21	218.51
3b	2.99	97.64	210.00
3c	2.44	97.64	210.00
3d	1.34	113.85	224.30
3e	-0.88	151.15	251.33
3f	3.81	110.78	235.56
3g	4.45	97.64	244.71

The first observation concerns the opposition between log P and PSA, which in fact is a good indicator of the reverse correlation between hydrophobicity (represented by log P) and polarity (represented by PSA). It can be observed that the lowest log P value is for compound **3e**, which has a free carboxyl group (log P = –0.88), and, conversely, has the highest PSA value (PSA = =151.15). Similarly, the highest log P value (4.45) is recorded for compound **3g**, correlated with the lowest PSA (97.64). Regarding the volumes of the molecules, of course that these values follow the molecular mass.

EXPERIMENTAL

Compounds **3a–3g** were obtained according to literature data.[17,18]

Cell Culture

The eukaryotic cell culture used, human ileocecal adenocarcinoma HCT-8 cells (ATCC CCL244™), were maintained as an adherent culture in Dulbecco's Modified Essential Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, USA) at 37 °C, 5% CO_2, in a humid atmosphere. The tested compounds were dissolved in DMF to prepare concentrated stock solutions (10mg/mL) prior to testing. Following, additional dilutions were prepared in culture medium.

MTS test

HCT-8 cells were seeded in 96-well plates at a density of 1×10^4 cells per well, incubated at 37°C for 24 hours, and treated with the tested compounds (ranging from 500 µg/mL to 0.24 µg/mL). After 24 hours, the viability of treated cells was measured using an MTS assay (Promega) following the manufacturer's protocol. Each experiment was performed three times. IC_{50} value was estimated by applying dose-response models using GraphPad Prism software (GraphPad Software Inc., SUA). OD values are presented in Supplementary Data.

Cell Cycle Analysis

To evaluate the influence of the tested compounds on the cell cycle, a number of 3×10^5 HCT-8 cells were treated with the tested compounds at a concentration of 50 μg/mL. The activity of compound 3**d** was also assessed at final concentrations of 20 μg/mL, 10 μg/mL, 5 μg/mL, and 1 μg/mL. After 72 hours of treatment, the cells were harvested and washed in a cold solution of PBS (pH 7.5), then fixed in cold ethanol (70%) and stored at – 20 °C overnight. The samples were then centrifuged, washed with PBS and re-suspended in 100 μL PBS. They were treated with RNase A (1 mg/mL), labeled with propidium iodide (100 μg/mL) and incubated in the dark at room temperature for 30 minutes before measurement. The DNA content of cells was quantified on a Beckman Coulter EPICS XL flow cytometer and analyzed using FlowJo 8.8.6 software (Ashland, Oregon, USA).

Apoptosis genes expression analysis

A number of 3×10^5 HCT-8 cells were treated with compounds **3a**, **3b**, **3c**, **3e**, **3f**, and **3g** at a final concentration of 50 μg/mL, and with compound **3d** at a final concentration of 20 μg/mL for 72 hours. Total RNA was extracted from treated cells with Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. For each sample, 2 μg of total RNA was used for reverse transcription with the High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystem), and 50 ng cDNA from each sample was used in real time PCR reaction. Real-time PCR was performed on an ABI 7300 Real Time PCR System using pre-validated Taqman Gene Expression Assays kits (Applied Biosystems): CASP3 (Hs00234387_m1), CASP7 (Hs00169152_m1), CASP8 (Hs00154256_m1), CASP9 (Hs00154261_m1), BAX (Hs00180269_m1), BCL2 (Hs00153350_m1). GAPDH was used as endogenous control. Each experiment was performed three times. Results were analyzed with RQ study software (Applied Biosystems). The $\Delta\Delta C_T$ method was used to compare the relative expression levels.

CONCLUSIONS

The cytotoxic effect of the compounds **3a–3g** on eukaryotic cells was determined. The most toxic compound proved to be **3d** which also slightly increased the S phase and decreased the G0/G1 phase in a dose dependent manner. The cytotoxic effect of the tested compounds **3a–3g** was also evaluated at the molecular level by analyzing the expression levels of some genes implicated in apoptosis. The compounds **3b**, **3c** and **3d** induced apoptosis through both, intrinsic and extrinsic, pathways, while compound **3a** activated only the intrinsic pathway.

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