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Synthesis of 7-Substituted Fluoroquinolone Derivatives Containing Triazolidine Dione Moiety and *In Vitro* Evaluation of their Cytotoxic Effects

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A series of fluoroquinolone derivatives holding triazolidine dione moieties have been synthesized and proved to be cytotoxic agents *in vitro* particularly against cancer cell lines (SKNMC, MCF7, A2780-CP, SW48, A549, KB, HT-29, $HepG_2$). The cytotoxic activity was assessed using MTT colorimetric assay. Our compounds showed less cytotoxicity than doxorubicin in all studied cell lines. The best results was obtained for the compound **3a** on A549 cell line (IC_{50} = 34.5 μM) and the compound **3b** on SW48 (IC_{50} = 42 μM) and A2780-CP (IC_{50} = 43 μM) cell lines. The compound **3b** that has the phenyl urazole moiety at C-7 position, showed better anticancer effect than the compound **3a** on SW48, A2780-CP and MCF7 cell lines.

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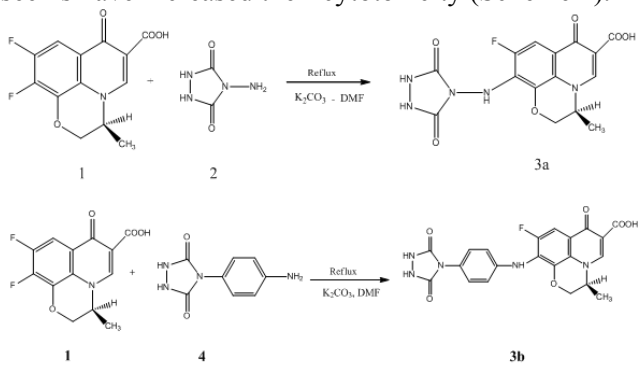
Introduction

Cancer is known medically as a malignant neoplasm, which includes of various diseases, all involving unregulated cell growth. There are over 200 different known cancers that afflict humans [1]. Quinolones are synthetic antibacterial compounds based on a 4-quinolone skeleton [2,3]. They have been developed for clinical use in human [4]. These antibiotics exert their effect by inhibition of two type II topoisomerase enzymes, DNA gyrase and topoisomerase IV [5,6]. DNA topoisomerases are found in both eukaryotic and prokaryotic cells and are target for chemotherapeutic intervention in antibacterial and anticancer therapies [7]. Topoisomerase II plays important roles in a number of fundamental nuclear processes [8] and is essential for the survival of eukaryotic cells[9]. Indeed, DNA topoisomerase II enzyme catalyzes the double-strand breakage of DNA to allow strand passage and thereby control the topology and conformation of DNA[10]. The activities of these drugs correlate with their ability to stabilize covalent enzyme-cleaved DNA complexes that are intermediates in the catalytic cycle of topoisomerase II[9]. Beyond its required physiological functions, the enzyme is a target for some of the most active compounds currently employed for the treatment of human cancers[8,11]. Among the topoisomerase II-targeted antineoplastic agents in clinical use are etoposide, amsacrine (mAMSA), adriamycin, and mitoxantrone. Since topoisomerase II-targeted drugs act by converting the enzyme into a cellular poison [9], antineoplastic potential is a reflection of the physiological level of the type II enzyme[12].

In view of the mechanistic similarities and sequence homologies exhibited by the prokaryotic type II topoisomerases and the eukaryotic type II topoisomerases, tentative efforts to selectively shift from an antibacterial to an antitumoral activity was made by synthesizing novel classes of quinolines [13,14]. The majority of quinolones in clinical use belong to the subset fluoroquinolones, which have a fluorine atom attached to the central ring system, typically at the C-6 or C-7 positions [7].

Fluoroquinolones have attracted much attention because of their broad spectrum of activity against various bacteria, mycobacteria and parasites [15]. Indeed, although fluoroquinolones are generally classified as broad-spectrum antibacterial agents, due to structural and functional similarities between bacterial DNA gyrase and mammalian topoisomerase II, the cytotoxicities of some of them also evaluated [16-18].

1-Acyl and 1,2-diacyl-1,2,4-triazolidine-3,5-diones were shown to be effective antineoplastic agents in both murine and human tissue cultured cell lines[19]. In the present study we have replaced the fluorine atom of C-7 position with 1,2,4-triazolidine-3,5-diones and investigated the cytotoxicity of the synthesized fluoroquinolone against eight cancer cell lines (KB, SKNMC, MCF7, HepG2, A2780-CP, SW48, A549, HT-29). The principal aim of our work is the discovery of novel cytotoxic and anticancer agents so in this study we want to achieve 1,2,4-triazolidine-3,5-diones -fluoroquinolone hybrids that seems have increased their cytotoxicity (Scheme 1).



Scheme 1. Synthesis of compounds **3a** and **3b**

Materials and Methods

Chemistry

All starting materials, reagents and solvents were purchased from Merck and Aldrich companies. The purity of the synthesized compounds was confirmed by thin layer chromatography (TLC) using various solvents of different polarities. Merck silica gel 60 F254 plates were used for TLC. ^{1}H-NMR spectra were recorded using a Bruker 250 spectrometer and chemical shifts are expressed as (ppm) with tetramethylsilane (TMS) as internal standard. The IR spectra were obtained on a Shimadzu 540 spectrophotometer (potassium bromide disks). Melting points were determined on a Kofler hot stage apparatus and are uncorrected.

Synthesis of 4-amino-1,2,4-triazolidine-3,5-dione (Urazine)

Dimethyl carbonate (0.25 mmol, 29.5 mL) and 31.25 mL hydrazine hydrate 80% (0.5 mol) were placed in a round bottomed flask equipped with a thermometer. The mixture was stirred until a single phase is formed. Heating continued for 4 hours, and the temperature was raised to 119 °C. The solution was cooled to 20 °C and allowed to stand for at least 1 h. The carbohydrazide crystals were separated by filtration and dried as completely as possible by suction. Then carbohydrazide (12.96 g, 0.144 mol) and 12 mL of HCl 12 M were mixed with a mechanical stirrer. The mixture was heated slowly on a hot plate with constant stirring. Heating was stopped when the temperature raised above 220 °C. The overall time of reaction was 4 hours. 10 ml of water was added to the cooled mixture and the urazine was filtered and washed with water, ethanol, and ether[20].

Yield: 62.5% (5.22 g); m.p. = 275 °C (decomposition); IR (KBr, cm^{-1}): ν_{max} 3510, 3474, 3410, 1724, 1650, 1625, 1450, 1180; ^{1}H-NMR (DMSO-d_6, 250 MHz): δ 9.83 (s, 2H, NH Amide), 4.77 (s, 2H, NH Amine) ppm.

Synthesis of 4-para-aminophenyl urazole

1-Ethoxycarbonyl-4-*para*-aminophenylsemicarbazide (35 g, 0.20 mol) was placed in 500-mL Erlenmeyer flask. The solution was warmed on a hot plate and stirred by a magnetic stirrer for 1.5 h. Then 80 mL of 4 M KOH was added to ensure that the reaction had taken place to a large extent. The hot solution was filtered by suction filtration. The filtrate was cooled in an ice bath and then acidified with concentrated hydrochloric acid (about 50 mL). A white solid precipitated, filtered, and then dried in vacuum desiccators at room temperature. Recrystallization from hot water (about 300 mL) was yielded a white crystalline compound in 100% yield (25.80 g) [21].

m.p. = 253-255 °C; IR (KBr, cm^{-1}): ν_{max} 3300, 3250, 2970, 2920, 1650, 1460, 1440, 1330; ^{1}HNMR (DMSO-d_6, 250 MHz): δ 10.21 (s, 2H, NH amide), 6.97-6.98 (d, J = 7.5 Hz, 2H, Arom), 6.51-6.58 (d, J = 7.5 Hz, 2H, Arom), 5.36 (s, 2H, NH Amine) ppm.

Typical procedure for the synthesis of (S)-10-((4-(3,5-dioxo-1,2,4-triazolidin-4-yl)amino)-9-fluoro-3-methyl-7-oxo-3,7-dihydro-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid (3a)

Potassium carbonate (1 g) was added to a solution of (*S*)-9,10-difluoro-3-mthyl-7-oxo-3,7-dihydro-2*H*-[1,4]oxazino[2,3,4-*ij*]quinoline-6-carboxylic acid **1** (0.141 g, 0.5 mmol), and 4-amino-1,2,4-triazolidine-3,5-dione **2** (0.116 g, 1 mmol) in DMF (5 mL), and the mixture was refluxed for 30 h. Then diluted hydrochloric acid (about 10 mL) was added to neutralize potassium carbonate. The solution was centrifuged to precipitate of compound **3a** and then dried in oven[2,4].

Yield: 85%; m.p. = 261 °C (decomposition); IR (KBr, cm^{-1}): ν_{max} 3448, 1720, 1624, 1477, 1307, 1165; ^{1}H-NMR (DMSO–d_6, 250 MHz): δ 14.87 (s, 1H, NH amine), 9.11 (s, 1H, CH, aromatic), 7.80-7.83 (d, J = 7.5 Hz, 1H, CH, aromatic), 5.01 (s, 1H, NH), 4.68-4.71 (d, J = 7.5 Hz, 1H, CH, aliphatic), 4.47-4.50 (d, J =7.5 Hz, 1H, CH, aliphatic), 2.73-2.89 (m, 1H, CH, aliphatic), 1.46-1.49 (d, J = 7.5 Hz, 3H, CH_3); MS (m/z, %): 377 (M^+, 12), 370 (33.6), 369 (92), 368 (100), 367 (28), 366 (17.6), 355 (26.4), 354 (39.2), 353 (49.6), 340 (32), 333 (52), 325 (30.4), 316 (36.8), 313 (71.2), 311 (43.2), 299 (47.2).

Typical procedure for the synthesis of (S)-10-((4-(3,5-dioxo-1,2,4-triazolidin-4-yl) phenyl)amino)-9-fluoro-3-methyl-7-oxo-3,7-dihydro-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid (3b)

Potassium carbonate was added to a solution of (*S*)-9,10-Difluoro-3-mthyl-7-oxo-3,7-dihydro-2*H*-[1,4]oxazino[2,3,4-*ij*]quinoline-6-carboxylic acid **1** (0.141 g, 0.5 mmol), and 4-*para*-aminophenyl urazole **4** (0.192 g, 1 mmol) in DMF (5 mL) and the mixture was refluxed for 36 h. Then diluted hydrochloric acid (about 10 mL) was added to neutralize potassium carbonate. The solution was centrifuged to precipitate of compound **3b** and then dried in oven[2,4].

Yield: 70%; m.p. = 297 °C (decomposition); IR (KBr, cm^{-1}): ν_{max} 3448, 1720, 1624, 1477, 1307, 1165; ^{1}H-NMR (DMSO–d_6, 250 MHz): δ 14.75 (s, 1H, NH Amine), 9.11 (s, 1H, CH, aromatic), 7.08-

7.95 (m, 5H, aromatic), 5.04 (s, 1H, NH), 4.67-4.70 (d, J = 7.5 Hz, 1H, CH, aliphatic), 4.47-4.50 (d, J = 7.5 Hz, 1H, CH), 2.73-2.89 (m, 1H, CH, aliphatic), 1.46-1.49 (d, J = 7.5 Hz, 3H, CH_3). MS (m/z, %): 453 (M^+, 8.1), 440 (15), 396 (26), 382 (31), 381 (32), 370 (47.2), 369 (99.5), 368 (100), 353 (72.4), 339 (76.4), 333 (55.2), 327 (43), 319 (47.2), 313 (99.5), 299 (81.3).

Biological activity

Cell culture: MCF7, NCBI-C135 (Human breast Adenocarcinoma), HepG2, NCBI-C158 (Human Liver Carcinoma), KB, NCBI-C152 (Human Mouth Carcinoma), SKNMC, NCBI-C535 (Human Brain Glioblastoma-astrocytoma), A2780-CP, NCBI-C454 (Human Ovary Carcinoma-Resistance to Cisplatin), SW48, NCBI-C480 (Human Colon Adenocarcinoma) A549, NCBI-C137 (Human Lung Carcinoma), HT-29, NCBI-C466 (Human colon carcinoma) were purchased from Pasture Institute of Tehran-Iran. Cell lines were grown and maintained in a humidified incubator at 37 ^oC with 5% CO_2 atmosphere. Cells were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), and antibiotics (100 IU/ml penicillin and 100 µl/ml streptomycin). Doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), penicillin and streptomycin were purchased from Sigma-Aldrich. The test compounds were dissolved in dimethylsulfoxide (DMSO), diluted with media and stored as the stock solutions with a concentration of 1.0 mg/mL at –20 ^oC (The concentration of DMSO was less than 1%)[13].

The synthesized compounds were tested against eight cell lines and compared to DMSO and doxorubicin as negative and positive controls respectively. Cells were seeded in 96-well plates at the density of 8000-10,000 viable cells per well and incubated for 24 h to allow cell attachment. The cells were then incubated for another 24-48 h (depends to cell cycle of each cell line) with various concentrations of compounds. Cells were then washed in PBS (Phosphate Buffer Saline) and 100 µL of fresh media and 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoli-umm bromide) solution (5 mg/mL) were added to each well. Additional 4 h of incubation at 37 °C were done and then the medium was discarded. Dimethylsulfoxide (60 µL) was added to each well and the solution was vigorously mixed to dissolve the purple formazan crystals. The absorbance of each well was measured by plate reader (Synergy 1; Biotech) at a wavelength of 540 nm. The amount of produced purple formazan is proportional to the number of viable cells. IC_{50} (µM) were calculated by Prism analysis, expressed in mean±SEM[13].

Results and Discussion

The *in vitro* cytotoxic activity of the test compounds **3a** and **3b** was investigated in comparison with doxorubicin against eight tumor cell lines using MTT colorimetric assay. The percentage of growth was evaluated *versus* controls not treated with test agents. For each compound, 50% inhibitory concentration (IC_{50}) was determined and reported in Table 1. The data for doxorubicin as a positive control was included for comparison. The compound **3a** in A549 cell line (IC_{50} = 34.5±0.1 µM) and **3b** in SW48 (IC_{50} = 42±0.30 µM) and A2780-CP (IC_{50} = 43±0.01 µM) cell lines showed the high tumor-specific cytotoxicity, indicating a new drug candidate for cancer chemotherapy (Figures 2,3,5). As we know most of the quinolones that showed anticancer activity contain aryl moiety at the C-7 position[4]. So, in our study the compound **3b** that has a bulk moiety in C-7 position, showed better anticancer effect. In other cell lines the remarkable cytotoxicity was not obtained (Figures 1,4,6-8). Our compounds showed less cytotoxicity than doxorubicin in all studied cell lines. The cytotoxic results for urazine 2, 4-*para*-aminophenyl urazole **4** and fluoroquinolone **1** were included and compared to doxorubicin. The IC_{50} values of triazolidine dione derivatives **2,4** and the starting fluoroquinolone **1** showed less IC_{50} than doxorubicin in studied cell lines (Table 1).

Table 1. IC_{50} (mean±SEM, µM) values of doxorubicin, triazolidine diones, and newly synthesized fluoroquinolone derivatives in different carcinoma cell lines after 48 h of exposure.

Compound	SKNMC	SW48	A2780-CP	MCF7	A549	HepG2	HT-29	KB
1	33.5±0.03	45±0.21	57±0.00	>250	>250	>250	>250	>250
2	67.3±0.18	50±0.34	65±0.02	>250	>250	>250	>250	>250
4	27.5±0.01	51±0.18	69±0.01	>250	>250	>250	>250	>250
3a	45±0.01	55±0.26	59±0.01	83±0.12	34.5±0.1	>250	>250	>250
3b	63±0.06	42±0.30	43±0.01	50±0.01	>200	>250	>250	>250
Doxorubicin	1±0.03	6.8±0.12	5.25±.015	4.76±0.4	1.7±0.78	4.3±0.65	5.6±0.7	1.2±0.5

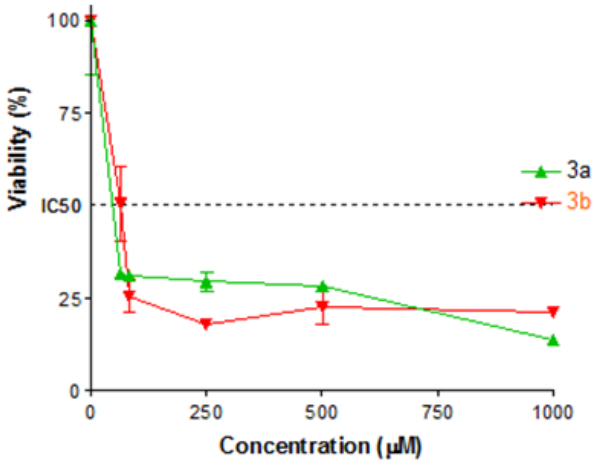


Fig. 1. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line SKNMC (Human Brain Glioblastoma-astrocytoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

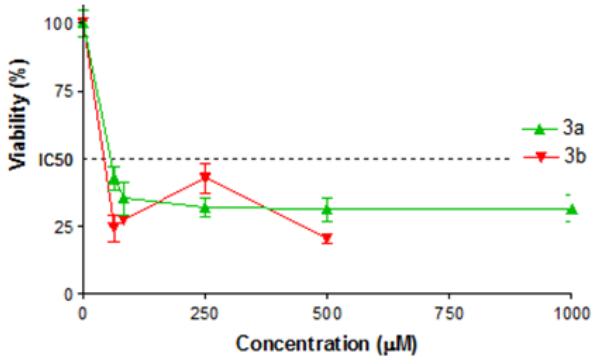


Fig. 2. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line SW48 (Human Colon Adenocarcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

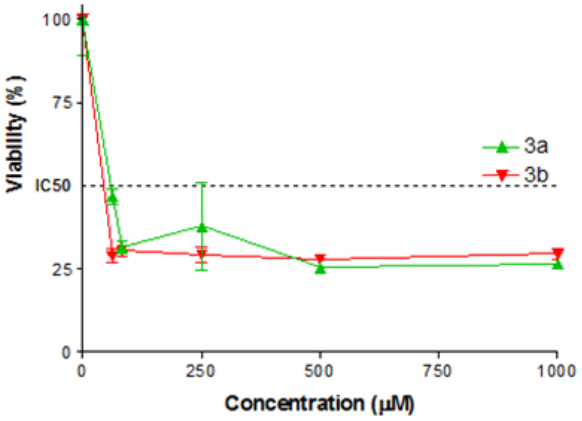


Fig. 3. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line A2780-CP (Human Ovary Carcinoma-Resistance to cisplatin). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

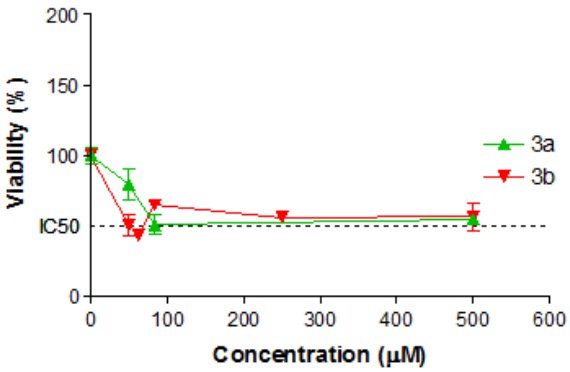


Fig. 4. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line MCF7 (Human breast Adenocarcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

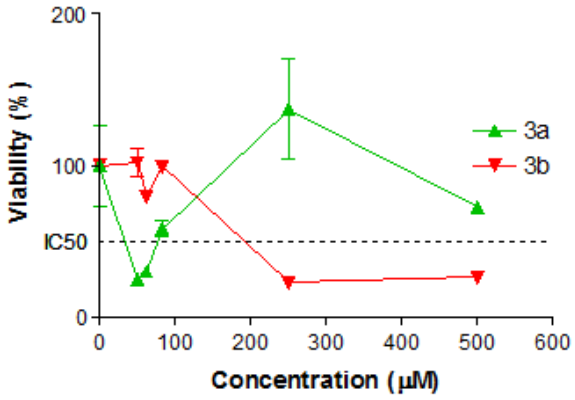


Fig. 5. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line A549 (Human Lung Carcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

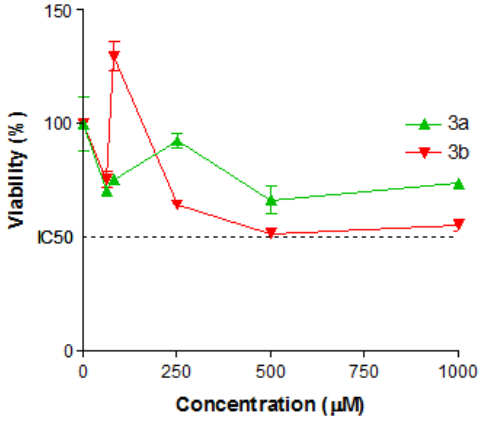


Fig. 6. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line HepG2 (Human Liver Carcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

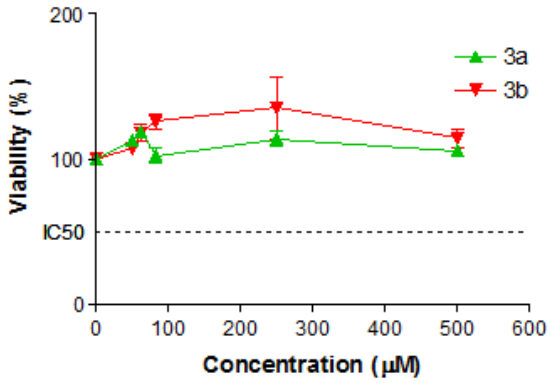


Fig. 7. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line HT-29 (Human colon carcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

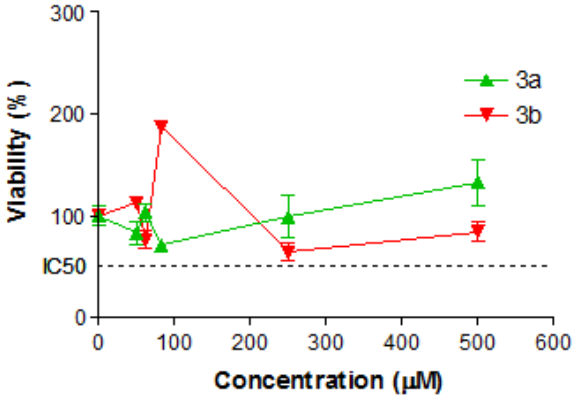


Fig. 8. The percentage of cytotoxicity versus concentration by MTT exclusion on cancer cell line KB (Human Mouth Carcinoma). IC_{50} value was obtained by plotting the $\log_{10}$ of the percentage of proliferation values versus drug concentrations. Data are expressed as the mean±SEM of three separate experiments (n = 3).

Conclusion

In summary, we have synthesized and evaluated a novel series of derivatives of fluoroquinolone-triazolidine dione hybrids with potential cytotoxic effects. The results obtained showed the test compounds had IC_{50} more than the control drug. Further studies are in progress to evaluate antimicrobial effects of the compounds on Gram-positive and Gram-negative bacteria.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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