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Original Article

Synthesis and cytotoxic evaluation of novel quinozalinone derivatives with substituted benzimidazole in position 3

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Abstract

Quinazolinone and benzimidazole are both fused heterocyclic compounds which have shown valuable biological properties including cytotoxic, antibacterial, and antifungal activities. In this study, a series of novel quinazolinone derivatives substituted with benzimidazole were synthesized in two parts. In the first part 2 - phenyl - 1*H* - benzimidazol - 6 - amine (**4**) was synthesized from the reaction of 4-nitro-o-phenylenediamine and benzoic acid. In the second part, new 3-(2-phenyl-1*H* benzoimidazol-5-yl)-3H-quinazolin-4-one derivatives (**8a-8f**) were also prepared. Finally compound **4** was reacted with the different benzoxazinone derivatives (**8a-8f**) to give the target compounds. The structures of the synthesized compounds were confirmed by IR and ^{1}HNMR. Cytotoxic activities of the final compounds were assessed at 100, 200, 300, 400, and 500 µM against MCF-7 and HeLa cell lines using the MTT colorimetric assay. Almost all compounds exhibited good cytotoxic activity against both cell lines. Compound **9d** demonstrated the highest cytotoxic activity against MCF7 and Hela cell lines with IC_{50} 70 µM and 50 µM, respectively.

Keywords: Cytotoxicity; Benzimidazole; MTT assay; Quinazolinone.

INTRODUCTION

Cancer is one of the major health problems in the world and is the second leading cause of death in developing countries (1). Despite the discovery of numerous drugs in the treatment of cancer and the significant advance in the treatment of this disease, most of the common treatments for this disease have encountered with serious problems including toxicity and drug resistance, so that research in this field has attracted the attention of many researchers (2).

Quinazolinones are heterocyclic compounds which have various biological effects including anticancer, sedative, anticompulsive, anti-inflammation, antibacterial, antifungal, anti-tuberculosis, antimalarial, antiviral, and anti HIV activities (3,4). Febrifugine (5), evodiamine (6), luotonin A (7), prazosin (8), methaqualone (9), and diproqualone (10) are some examples of quinazolinone-based drug which have good therapeutic effects. Gefitinib and erlotinib are the most important anticancer drugs in this category (11). Anticancer effect of quinazolinone derivatives is mainly attributed to their multi target activities including inhibition of topoisomerase I, EGFR tyrosine kinase, and dihydrofolate reductase inhibition (12-15).

Benzimidazoles are important class of heterocyclic compounds which have a wide range of therapeutic effects such as anti-inflammation, antimicrobial, antiviral, anti-fungal, antihypertension, antihistamine, and anticancer activities (16-19).

Omeprazole, thiabendazole, norastemizole, and telmisartan are some of the well-known examples of drugs containing benzimidazole pharmacophore possessing divert pharmacological activities (20).

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According to the previous studies, there are many anticancer drugs based on benzimidazole nucleus including nocodazole (NSC-238189) which acts by interfering with microtubule polymerization. This drug is a potent inhibitor of various cancer-related kinases including AB1, C-KIT, BRAF, MEK-1, MEK-2, and MET. Methyl-2-benzimidazole carbamate (carbendazim, FB642) is another example of benzimidazole-based drug which induces apoptosis by microtubule function inhibition. Veliparp (ABT-888) acts as an inhibitor of poly (adp ribose) polymerase (PARP) which is a target in breast cancer cell lines (21).

According to the biological activities of imidazole and quinazolinone derivatives we were interested in the synthesis of new hybrid compounds bearing these two pharmacophores in a single chemical framework.

MATERIALS AND METHODS

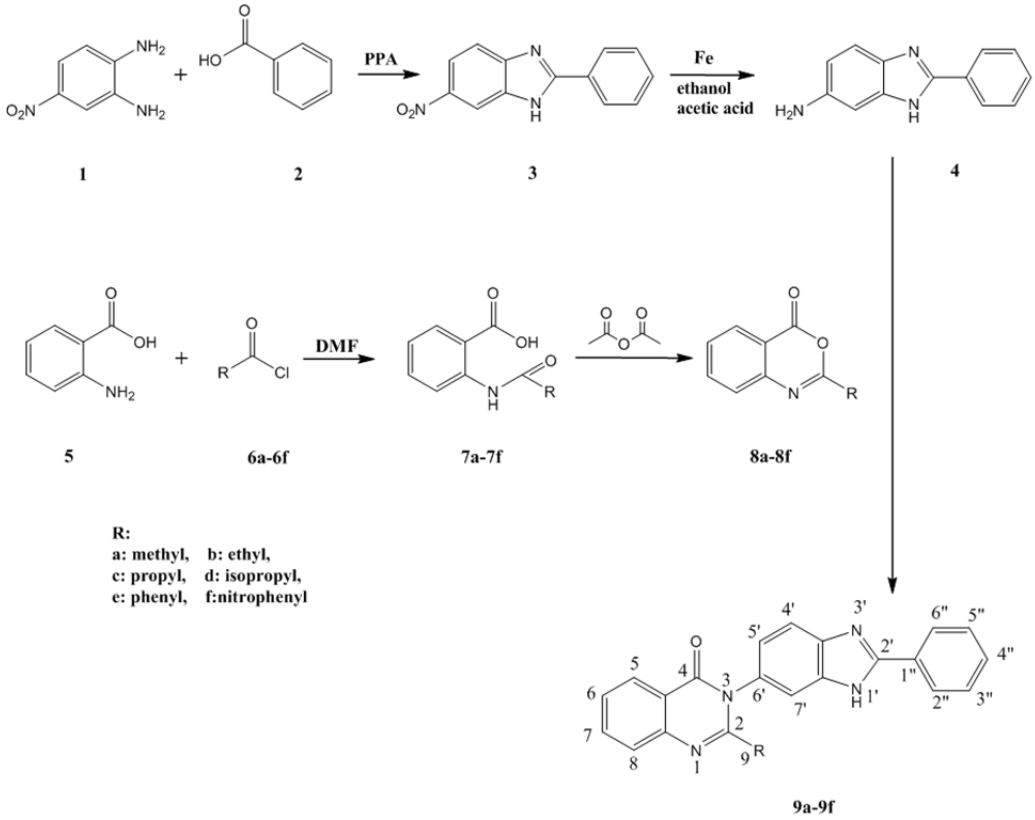
Instrumentation

All chemicals, solvents, and reagents were supplied from commercial suppliers such as Merck (Germany) and Aldrich (USA). Proton nuclear magnetic resonance (^{1}HNMR) spectra of synthetized compounds were determined using (Bruker 400 MHz, Germany) spectrometer, and chemical shifts were shown as δ (ppm) with tetramethylsilane (TMS) as the internal standard. Melting points were recorded by utilizing electro thermal melting point analyzer apparatus (IA 9000, UK) and are uncorrected. The infrared (IR) spectra were obtained on a Shimadzu 470 spectrophotometer (Japan; potassium bromide disks). Cell lines were purchased from Pasteur Institute of Iran, Tehran, I.R. Iran.

Preparation of compounds

3 - (2 - Phenyl - 1 *H* - benzoimidazol - 6-yl) quinazolin-4(3H)-one derivatives (**9a-9f**) were prepared from two separate reaction steps to produce the benzimidazole **4** and benzoxazinone derivatives **8a-8f,** respectively.

In the first part, 6-nitro-2-phenyl-1*H*-benzoimidazole (**3)** (Scheme 1) was prepared and then reduced to phenyl-1*H*-benzoimidazol-6-amine (**4)**.



Scheme 1. General reaction scheme for preparation of the final compounds.

In the second part a group of benzoxazinone derivatives with different substituents at position 2 were synthesized. Finally the primary amine **4** was reacted with the benzoxazinones **8a-8f** to produce target compounds **9a-9f** as explained below.

Procedure for the preparation of 5-nitro-2-phenyl-1H-benzo imidazole (3)

4 - Nitro - o - phenylenediamine (3.22 g, 21 mmol) was mixed with benzoic acid (2.44 g, 20 mmol) and stirred in poly-phosphoric acid for 5 h at 120-150 °C. The reaction was quenched with water and the pH was increased to 6 using saturated NaOH solution. The product was filtered and the separated cake was washed with water and dried to deposit a solid. The solid was dissolved in hot ethyl acetate and filtered to remove some solid impurities (22). The solvent was removed under reduced pressure and the resulted solid was recrystallized from water and isopropanol (5:1) to give the final product **3.**

Procedure for the preparation of the 2-phenyl-1H-benzo imidazol-5-amine (4)

A suspension of 5-nitro-2-phenyl-1*H*-benzo imidazole (1.19 g, 5 mmol) and iron powder (1.1 g, 20 mmol) in aqueous ethanol (120 mL, 70% v/v) containing acetic acid (2 mL, 30 mmol) was refluxed for 2 h. The solution was filtered after cooling to remove the catalyst. The solvent was then evaporated under reduced pressure to give the final product 2-phenyl-1H-benzo imidazol-5-amine **4** (22).

Procedure for the preparation of benzoxazinones (8a-8f)

To a solution of antranilic acid (1.37 g, 10 mmol) in dimethylformamide (5 mL) different acyl chlorides **6a-6f** (15 mmole) were added dropwise and the resulting solutions were stirred for 3 h. The end of the reactions was determined by thin layer chromatography (TLC). The mixtures were then poured into distilled water and stirred for additional 1 h. Finally the precipitated products were collected by filtration and washed with water to furnish **7a-7f** (23). Each compound of the previous step (**7a-7f**) (2.5 mmol) was added to acetic anhydride (2 mL) and refluxed at 140 °C until the starting materials **7a-7f** were disappeared from TLC. At the end of the reaction, the excess of acetic anhydride was removed from the reaction medium under reduced pressure. The resulting products were cooled to give solid mass. Finally, the products were washed with hexane to give benzoxazinones (**8a-8f**) (24).

Procedure for the preparation of 3-(2-phenyl-1H benzoimidazol-5-yl)-3H-quinazolin-4-one derivatives (9a-9f)

A mixture of compound **4** (0.418 g, 2 mmol) and compounds **8a-8f** (1 mmol) were refluxed for a period of 4-6 h in glacial acetic acid (20 mL). The reaction progression was investigated using TLC and at the end of the reaction, acetic acid was removed using rotary evaporator to give target compounds **9a-9f** (24).

Cell culture conditions

Cytotoxic activity of target compounds was assessed against HeLa and MCF-7 cells. HeLa and MCF-7 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI) with 5% v/v fetal bovine serum and 1% penicillin/streptomycin antibiotic solution. The cultured cells were maintained at 37 °C in a humidified atmosphere (90%) containing 5% CO2. The medium was replaced every two to three days and sub-cultured when the cell population density reached to 70-80% (25).

Cytotoxicity assay

HeLa and MCF-7 cells were seeded in 96-well tissue culture plates at a concentration of 5×10^4 cells/µL and incubated overnight. Then, cells were treated with different concentrations of the target compounds for 48 h (final concentrations in the wells were 10 (only for HeLa), 100, 200, 300, 400, and 500 µM). At the end of the incubation period, the medium was removed and 20 µL of the MTT solution (5 mg/mL in PBS) was added to each well and incubated for further 3 h. Finally, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO)

and absorbance of each well was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (25). Cell survival was calculated using the following equation:

$$\%Cell\ survival = \frac{Absorbance\ of\ treated\ well - absorbance\ of\ blank}{Absorbance\ of\ control\ well - absorbance\ of\ blank} \times 100$$

The half maximal inhibitory concentration (IC_{50}) values were determined by plotting the cell survival against compound concentrations.

Statistical analysis

One-way analysis of variance (ANOVA) followed by LSD post hoc test were used for data analysis. All results were expressed as mean ± SEM. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the SPSS Statistics 24.

RESULTS

5-Nitro-2-phenyl-1H-benzo imidazole (3)

Pale yellow powder, yield 80%, m.p: 204-207 °C, lit. m.p 206-208 °C ref (22). ^{1}HNMR (400 MHz: DMSO-d_6): 7.64-7.56 (3H, m, H-C$^{3'}$, H-C$^{4'}$, H-C$^{5'}$), 7.78 (2H, S, H-C^4), 8.14 (1H, dd, J = 6.8 Hz, J = 2 Hz, H-C^5), 8.23 (3H, dd, J = 6.0 Hz, J = 1.6 Hz, H-C$^{2'}$, H-C$^{6'}$), 8.49 (1H, S, H-C^7).

2-Phenyl-1H-benzo imidazol-5-amine (4)

Brown powder, yield: 60%, m.p: 290-295, lit. m.p 292-293 ref (22). ^{1}HNMR (400 MHZ: DMSO-d_6): 4.85 (2H, s, NH_2), 6.50 (1H, d, J = 7.2 Hz, H-C^5), 6.69 (1H, s, H-C^7), 7.26-7.47 (4H, m, H-C^4, H-C$^{3'}$, H-C$^{4'}$, H-C$^{5'}$), 8.10 (2H, d, J = 6 Hz, H-C$^{2'}$, H-C$^{6'}$).

2-Methyl-3-(2-phenyl-1H-benzoimidazol-6-yl) quinazolin-4(3H)-one (9a)

Pale brown powder, yield: 55%, m.p: 112-114 °C. IR (KBr, cm^{-1}) v_{max} = 3422 (NH), 3025(C-H, Ar), 2926 (C-H), 1681 (C=O), 1597 (C=C).^{1}HNMR (400 MHz: DMSO-d_6): 2.28 (3H, s, H-C^9), 7.28 (1H, dd, J = 6.4 Hz, J = 2.0 Hz, H-C^8), 7.61-7.68 (4H, m, H-C^6, H-C$^{3"}$, H-$^{4"}$, H-C$^{5"}$), 7.77-7.85 (3H, m, H-C$^{4'}$, H-C$^{5'}$, H-C$^{7'}$), 7.95 (1H, t, J = 6.8 Hz, H-C^7), 8.22 (1H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C^5), 8.36 (2H, dd, J = 7.2 Hz, J = 1.2 Hz, H-C$^{2"}$, H-C$^{6"}$).

2-Ethyl-3-(2-phenyl-1H-benzoimidazol-6-yl) quinazolin-4(3H)-one (9b)

Cream powder, yield: 45%, m.p: 109-110 °C. IR (KBr, cm^{-1}) v_{max} = 3418 (NH), 3067(C-H, Ar), 2931(C-H), 1665 (C=O), 1590 (C=C).^{1}HNMR (400 MHz: DMSO-d_6): 0.906 (3H, t, J = 7.2 Hz, H-C^{10}), 1.76-1.82 (2H, m, H-C^9), 7.26 (1H, d, J = 6.4 Hz, H-C^8), 7.59-7.68 (4H, m, H-C^6, H-C$^{3"}$, H-C$^{4"}$, H-C$^{5"}$), 7.77-7.83 (3H, m, H-C$^{4'}$, H-C$^{5'}$, H-C7'), 7.96 (1H, t, J = 6.8 Hz, H-C^7), 8.23 (1H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C^5), 8.35 (2H, dd, J = 7.2 Hz, J = 1.2 Hz, H-C$^{2"}$, H-C$^{6"}$).

3-(2-Phenyl-1H-benzoimidazol-6-yl)-2-propyl -quinazolin-4(3H)-one (9c)

Brown powder, yield: 48%, m.p: 115-117 °C. IR (KBr, cm^{-1}) v_{max} = 3407 (NH), 3071 (C-H, Ar), 2928 (C-H), 1659 (C=O), 1583(C=C). ^{1}HNMR (400 MHz: DMSO-d_6): 0.81 (3H, t, J = 7.6 Hz, H-C^{11}), 1.69 (2H, hex, J = 7.2 Hz, H-C^{10}), 2.36-2.39 (2H, m, H-C^9), 7.19 (1H, dd, J = 6.4 Hz, J = 2 Hz, H-C^8), 7.51-7.60 (4H, m, H-C^6, H-C$^{3"}$, H-C$^{4"}$, H-C$^{5"}$), 7.69-7.75 (3H, m, H-C$^{4'}$, H-C$^{5'}$, H-C$^{7'}$), 7.86 (1H, t, J = 6.8 Hz, H-C^7), 8.135 (1H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C^5), 8.241 (2H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C$^{2"}$, H-C$^{6"}$).

2-Isopropyl-3-(2-phenyl-1H-benzoimidazol-6-yl) quinazolin-4(3H)-one (9d)

Cream powder, yield: 50%, m.p: 120-123 °C. IR (KBr, cm^{-1}) v_{max} = 3421(NH), 3067 (C-H, Ar), 2929 (C-H), 1682 (C=O), 1590 (C=C).^{1}HNMR (400 MHz: DMSO-d_6): 1.16-1.19 (6H, m, H-C^{10}, H-C^{11}), 2.66-2.72 (1H, m, H-C^9), 7.23 (1H, dd, J = 6.4 Hz, J = 2.0 Hz, H-C^8), 7.51-7.60 (4H, m, H-C^6, H-C$^{3"}$, H-C$^{4"}$, H-C$^{5"}$), 7.70-7.76 (3H, m, H-C$^{4'}$, H-C$^{5'}$, H-C$^{7'}$), 7.86 (1H, t, J = 6.8 Hz, H-C^7), 8.13 (1H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C^5), 8.246(2H, d, J = 6.8 Hz, H-C$^{2"}$, H-C$^{6"}$).

2-Phenyl-3-(2-phenyl-1H-benzoimidazol-6-yl) quinazolin-4(3H)-one (9e)

Pale brown powder, yield: 38%, m.p: 134-137 °C. IR (KBr, cm^{-1}) v_{max} = 3405(NH),

3067 (C-H, Ar), 2932 (C-H), 1527 (C=C, Ar).[1]HNMR (400 MHZ: DMSO-d_6): 7.20 (1H, dd, J = 6.8 Hz, J = 1.6 Hz, H-C^{8}), 7.247 (3H, t, J = 3.6Hz, H-C^{11}, H-C^{12}, H-C^{13}), 7.49-7.61 (6H, m, H-C^{10}, H-C^{14}, H-C$^{3"}$, H-C$^{4"}$, H-C$^{5"}$, H-C$^{4'}$), 7.65-7.69 (2H, m, H-C^{6}, H-C$^{7'}$), 7.86 (1H, d, J = 8.4 Hz, H-C$^{5'}$), 7.97 (1H, t, J = 7.2 Hz, H-C^{7}), 8.23 (2H, d, J = 7.2 Hz, H-C$^{2"}$, H-C$^{6"}$), 8.29 (1H, dd, J = 6.8 Hz, J = 1.2 Hz, H-C^{5}).

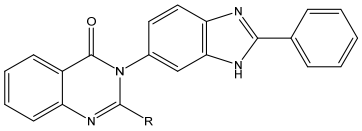
2-(4-Nitrophenyl)-3-(2-phenyl-1H-benzo-[d]imidazol-6-yl) quinazolin-4(3H)-one (9f)

Pale yellow powder, yield: 30%, m.p: 129-132 °C. IR (KBr, cm^{-1}) v_{max} = 3419 (NH), 3071 (C-H, Ar), 2928 (C-H), 1689 (C=O), 1581 (C=C), 1348, 1433 (NO2). [1]HNMR (400 MHz: DMSO -d_6): 7.31 (1H, d, J = 7.2 Hz, H-C$^{4'}$), 7.59-7.66 (4H, m, H-C7', H-C$^{3"}$, H-C$^{4"}$, H-C$^{5"}$), 7.76-7.79 (2H, m, H-C^{6}, H-C$^{5'}$), 7.88 (2H, d, J = 8.4 Hz, H-C$^{2"}$, H-C$^{6"}$), 7.95 (1H, d, J = 8.4 Hz, H-C^{8}), 8.063 (1H, t, J = 6.8 Hz, H-C^{7}), 8.19 (2H, d, J = 8.8 Hz, H-C^{10}, H-C^{14}), 8.30 (2H, d, J = 7.2 Hz, H-C^{11}, H-C^{13}), 8.38 (1H, d, J = 8.0 Hz, H-C^{5}).

Cytotoxic effect of synthesized compounds

IC_{50} of target compounds against MCF-7 and HeLa cell lines are listed in Table 1. Compounds **9a-9f** showed significant toxic effect ($P < 0.5$) compared with positive control group in both cell lines (Figs. 1 and 2).

Table 1. The IC_{50} ± SD values (μM) of compounds **9a-9f** against MCF-7 and HeLa cell lines using MTT assay.



Target compounds	R	IC_{50} (μM)	
		MCF-7	HeLa
9a	Methyl	110 ± 7.9	180 ± 5.8
9b	Ethyl	130 ± 4.3	150 ± 11.2
9c	Propyl	115 ± 5.6	80 ± 9.6
9d	Isopropyl	70 ± 8.6	50 ± 4.6
9e	Phenyl	190 ± 3.5	> 250
9f	Nitro phenyl	> 250	> 250
Doxorubicin (24)	-	3.12	3.56

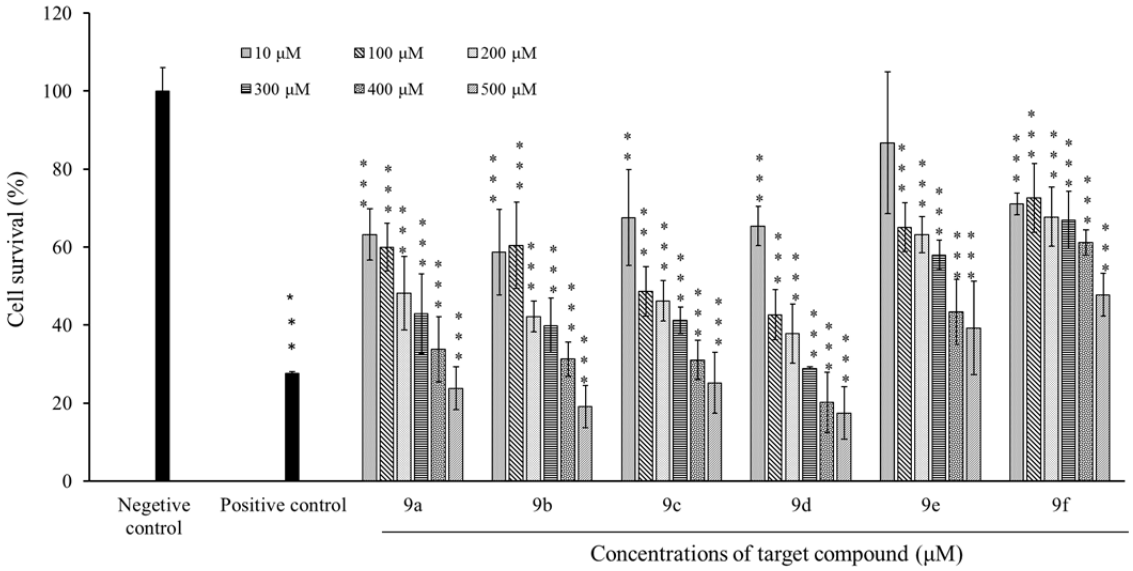


Fig. 1. Cytotoxic effects of compounds **9a-9f** on HeLa cells following exposure to different concentrations (μM) of compounds **9a-9f**. Cell survival was determined using the MTT method. Data are presented as mean ± SD of cell survival compared to negative control; $^{**}P < 0.01$, $^{***}P < 0.001$; n = 3. Positive control, doxorubicine 1 μM.

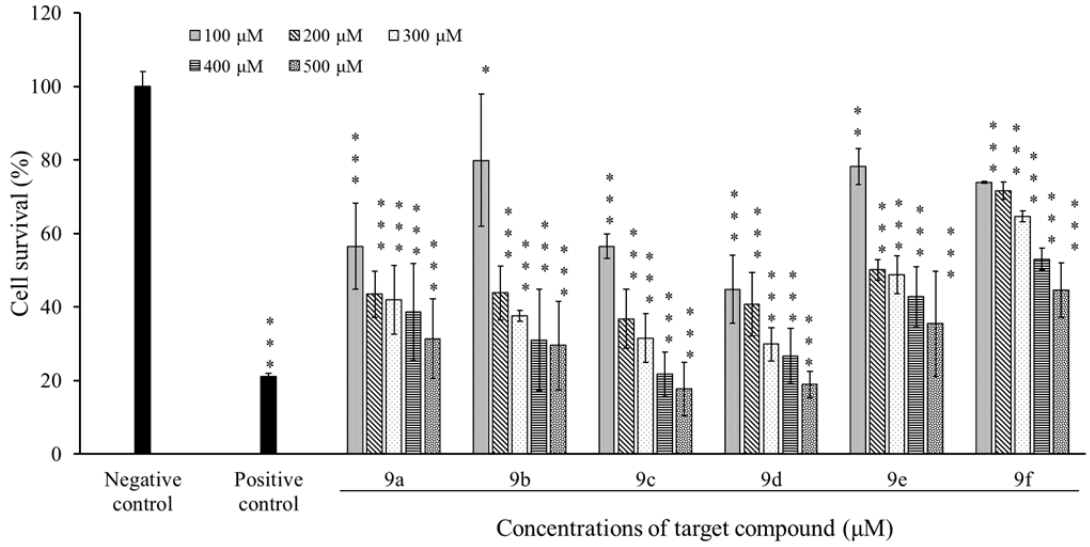


Fig. 2. Cytotoxic effects of compounds **9a-9f** on MCF-7 cells following exposure to different concentrations (µM) of compounds **9a-9f**. Cell survival was determined using the MTT method. Data are presented as mean ± SD of cell survival compared to negative control; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; n = 3. Positive control, doxorubicine 1 µM.

DISCUSSION

In this study, we synthesized some new quinazoline derivatives with substituted benzimidazole at position 3. Amine-bearing benzimidazole moiety (**4**) was synthesized via 2 steps. In the first step, condensation of 5-nitro-o-phenylenediamine and benzoic acid was performed in polyphosphoric acid which facilitated the removal of water by activating the acidic group. In the second step, reduction of the nitro group to amine was carried out using iron in the mixture of ethanol and acetic acid.

Benzoxazinones are highly reactive and should be used immediately after preparation (26). In this study, benzoxazinones were prepared in two parts. In the first part, anthranilic acid was treated with the acyl chlorides to prepare N-acyl anthranilic acids. These compounds could be prepared easily at room temperature via a nucleophilic substitution reaction (27). This could be because of the high electrophilicity of the carbonyl group next to a powerful electron withdrawing group (Cl) in acyl chloride. In addition dimethylformamide could also facilitate this reaction by removal of hydrogen from the amino group (28). Subsequent reflux of N-acyl anthranilic acids in acetic anhydride resulted in production of the corresponding benzoxazinones via a dehydrative cyclization mechanism. The excess amount of acetic anhydride was removed immediately to avoid its side reactions with primary amines utilizing in the next step to prevent the production of corresponding amide by product.

Finally, compounds **8a-8f** were added to **4** to produce final hybrids via a nucleophilic substitution mechanism.

Looking at the ^{1}HNMR spectra of **4** and the target compounds **9a-9f** a singlet peak at 4.855 belonging to the NH_2 group of **4** is obvious while after the reaction of **4** with the benzoxazinons this peak was completely disappeared in the ^{1}HNMR spectra of the target compounds to confirm that the reaction was undertaken.

Cytotoxic effects of the synthesized hybrids were investigated on HeLa and MCF-7 cells by MTT assay. According to the results shown in Table 1, compounds **9e** and **9f** bearing aromatic substituents on C_2 of the quinazolinone ring showed lowest cytotoxic activities on both cell lines, i.e. IC_{50} > 250 µM, while compounds **9a-9d** containing aliphatic substituents on C_2 were more active on both cell lines with IC_{50} values between 50-150 µM. It seems that the presence of electron donating substituents on C_2 could be

in favor of the activity for these compounds while electron withdrawing groups have opposite effects, perhaps because of un favored electronic effects on the site of action. A novel series of quinazolin-4-one benzimidazoles were developed recently by Singla *et al*. some of which showed promising dihydrofolate reductase inhibitory activity in an enzyme immunoassay test (29). Another set of quinazolin-benzimidazole hybrids reported by Sharma *et al*. were also exhibited remarkable activity against several cancer cell lines including MCF-7 (30). The quinazolinone and benzimidazole hybrids could be considered as useful templates for future development and modifications to obtain more potent compounds.

CONCLUSION

In summary, the novel derivatives of quinazolinone with substituted benzimidazole at position 3 were synthesized in several steps and their *in vitro* cytotoxic activities were evaluated against MCF-7 and HeLa cell lines. The cytotoxic evaluation of synthesized derivatives on both MCF-7 and HeLa cell lines represented that compounds with phenyl and nitrophenyl substitutes had the lowest cytotoxic activity against both cell lines and compound **9d** with isopropyl substituent had the highest potency.

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