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ORIGINAL ARTICLE

Synthesis, Cytotoxicity Evaluation and Molecular Docking Simulation of Some New 4-(3H)-Quinazolinone -Thiadiazole Hybrids as Anticancer Agents

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ABSTRACT
A new series of quinazolinone compounds (I–IV) incorporating 1,3,4-thiadiazole moiety were synthesized .The compounds were characterized by ATR-FTIR,[1]HNMR and [13]CNMR. Their in vitro antitumor efficacy was evaluated against two cancer cell lines, a breast cancer cell line (MCF-7), and lung cancer cell line (A549) using MTT assay .Methotrexate (MTX) was used as a positive control. In order to evaluate the selectivity of the tested compounds toward cancer cells, they were tested also against the normal cells using epithelial cells derived from normal human fibroblast (NHF). All the tested compounds showed less toxicity against (NHF) cell lines compared with cancer cell lines. Among the tested compounds, compound I showed the most potent antitumor activity against MCF-7cell lines (IC50:7.832 µM), the comparative IC50 value for methotrexate on these cell lines was (27.325 µM). All compounds exhibited higher antitumor activity against A549 cell lines than methotrexate, the most potent among them was compound III (IC50:6.669µM). The binding pattern of the designed compounds with their target protein (EGFR kinase) were studied by molecular docking study using GOLD software.
Keywords: 4(3H)-quinazolinone, EGFR, MCF-7, Molecular docking**.**

INTRODUCTION
Despite the advances in the techniques followed in cancer therapy such as immunotherapy and gene therapy, the treatments of choice still depend on surgery, radiotherapy and chemotherapy [1]. The main limitations of conventional chemotherapeutic agents attributed to their high toxicity to normal cells at therapeutic doses and the development of resistance to the drug by the cancer cells, therefore the development of highly potent and more selective anticancer drugs is an attractive target in modern medicinal chemistry [2] . Resistance to anticancer drug therapy attributed to several factors such as the individual variations among patients and genetic differences in tumor cells [3]. Epidermal growth factor receptor (EGFR) is a key mediator playing a vital role in the regulation of several cellular processes including proliferation, migration and survival [4] **,** it has been termed as a proto-oncogene [5]. In the pathological conditions, mostly in breast and lung cancer, EGFR is found to act as a driver of tumorigenesis [6]. EGFR has been found to overexpress in different types of tumors such as prostate, breast, colon and ovarian cancers, inhibition of this enzyme was associated with apoptosis induction in several types of tumors mainly in lung and breast cancers [7]. Quinazoline heterocyclic moiety resembles both the purine and pteridine nuclei and possess a wide spectrum of biological effects such as anticancer, antiviral and anti-inflammatory activities [8]. Quinazolinone scaffold has drawn much attention in the field of drug design and development due to its wide range of biological applications [9]. Different mechanisms have been proposed for the anticancer activity of quinazolinone-based structures such as inhibition of the DNA repair enzymes, inhibition of many enzymes essential in cell division such as thymidylate synthetase (TS) and (EGFR) tyrosin kinase enzymes [10][11]. Some reported EGFR inhibitors bearing quinazolinone nucleus are shown in Figure (1) [12].

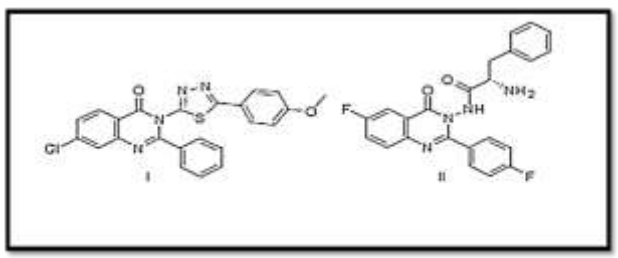


Figure 1: Some reported EGFR inhibitors containing quinazolinone nucleus.

On the other hand, several studies have reported the importance of 1,3,4-thiadiazole as a promising moiety in the designing of antitumor drugs against many cancer cell lines via the inhibition of many biological targets such as histone deacetylase and tyrosine kinase [13] **.** The sulfur atom of the thiadiazole ring has been found to improve the lipophilicity that enable the compounds to penetrate the cell membranes and bind to their targets with high affinity [14] [15]. Hybridization of two different biologically active scaffolds in one molecule is widely used strategy in the development of new compounds to achieve a synergistic effect. [16]

MATERIALS AND METHODS
Chemical synthesis: 5-amino-1,3,4-thiadiazole-2-thiol was purchased from Sigma-Aldrich (Switzerland), 6-(bromomethyl)-2-methyl-4(3H)-quinazolinone was purchased from Henan Tianfu Chemical Co. (china), 1-Hydroxybenzotriazole (HoBt) was purchased from merck (Germany) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl) was purchased from Sigma-Aldrich (Germany), the progress of the reactions and purity of the products were monitored by thin-layer chromatography (TLC) on precoated aluminium sheets Silica Gel Merck 60 F254 in different solvent systems , the spots were visualized by using a 254nm UV lamp. Melting points of all the synthesized compounds were determined by open capillary method using Stuart SMP3 apparatus (UK). IR Spectra were performed by utilizing Shimadzu model spectrometer (Japan) and the Specac® ATR diamond sort (UK) where spectra are listed between 4,000–400 cm^{-1} ranges. ^{1}H-NMR and ^{13}C-NMR analysis were performed using the Bruker and Varian model spectrometer at 500 MHz for^{1}H-NMR and 125 MHz for^{13}C-NMR with tetramethylsilane (TMS) as internal standard, DMSO- d6 as the solvent and the chemical shift values were given in δ (ppm) scale and the signals were described as s (singlet), d (doublet), t (triplet), m (multiplet), br (for broad signals) whereas coupling constants (J) were expressed in hertz.

Chemical synthesis of 6-(((5-amino-1,3,4-thiadiazol-2-yl)thio)methyl)-2-methylquinazolin-4(3H)-one (A: To a mixture of 5-amino-1,3,4-thiadiazole-2-thiol (1.28g,9.61mmol) in dry DMF (10ml) , anhydrous potassium carbonate (1.8g,12mmol) was added and the mixture stirred for 15 min., then a solution of 6-(bromomethyl)-2-methyl-4(3H)-quinazolinone (2.43g,9.61mmol) in dry DMF (10ml) has been added gradually , the mixture was then stirred at 45°C for 36hrs.The reaction progress was checked by TLC using acetone: cyclohexane (3:2) as a solvent system. At the end of reaction, the mixture was filtered and the filtrate was poured

into cold water, the product was precipitated, filtered off, washed with distilled water (3x50ml), dried then recrystallized from (ethanol:DMF) mixture (4:1). Yellow solid, yield: 52%, m.p (263-265 °C).R_f = 0.25 (acetone:cyclohexane 3:2), ATR-FTIR (u, cm^{-1}) :3329 and 3298 (NH_2) str., 3124 (NH) str.of sec.amide , 3047 Ar (C-H) , 1689 (C=O) str. of amide ,1608(C=N) str. ^{1}H NMR (500 MHz, DMSO-d6, δ =ppm):2.33 (s, 3H, quinazolinone-CH_3),4.41(s,2H-CH_2-S-),7.27(br s,2H,NH_2),7.52(d,1H,J=8.37,Ar-H),7.73(d, 1H,J=8.37,Ar-H,),8.02(s,1H,Ar-H).12.19(br s,1H, NH) .^{13}CNMR(125MHz,DMSO-d6): 21.88, 38.41,120.84, 126.25, 127.29, 135.56, 135.60, 148.71, 149.46, 154.79,161.93,170.41.

Chemical synthesis of the target compounds (I-IV): Benzoic acid or one of its p-substituted derivatives (4.0mmol) was dissolved in dry DMF (5ml) and the mixture was cooled down with ice bath to 0 ^{0}C then 1-Hydroxybenzotriazole (HoBt) (5mmol) and (EDC-HCl) (5mmol) were added ,the mixture was stirred on ice bath for 2hrs.(the disappearance of the acid and appearance of the activated ester was checked by TLC).Then a solution of compound A (4.0mmol) dissolved in dry DMF (10ml) has been added gradually to the stirred reaction mixture with keeping temperature at 0^{0}C. After completing the addition, the mixture left to stir at room temperature for 48hrs.The product was precipitated, collected by filtration and purified by washing with HCl (5%) (2x30ml), $NaHCO_3$ (5%) (2x30ml) and distilled water (3x50ml) then dried.

The following compounds has been synthesized using the general procedure mentioned above:

Synthesis of N-(5-(((2-methyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl)thio)-1,3,4-thiadiazol-2-yl)benzamide (I): Yellow fluffy powder, yield (48%), m.p (335-337^{0}C), $R_{f=}$ 0.44 (n-hexane: ethylacetate: methanol 5:3:2). ATR-FTIR (u, cm^{-1}), 3174(NH) str.of sec.amide, 3013 Ar (C-H), 1678(C=O) str. of amide, 1616 (C=N) str. ^{1}HNMR (500 MHz, DMSO-d6, δ=ppm): 2.34(s, 3H, quinazolinone-CH_3),4.66(s,2H-CH_2-S-),7.56(m,3H,Ar-H),7.67(t,1H,Ar-H),7.83(d,1H J= 8.39 ,Ar-H),8.11(d,2H,J=8.67,Ar-H),8.13(s,1H,Ar-H),12.22(br s,1H,NH),13.14 (br s, 1H, NH) .^{13}CNMR (125MHz,DMSO-d6): 21.90 ,37.44 , 121.34, 126.33 , 127.37 ,128.87 ,129.14 ,133.58, 135.21, 135.58, 148.79, 154.89,161.93.

Synthesis of 4-chloro-N-(5-(((2-methyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl)thio)-1,3,4-thiadiazol-2-yl)benzamide(II): White fluffy powder, yield (51%) ,m.p (338-341^{0}C) R_f=0.41(n-hexane :ethylacetate :methanol 5:3:2).ATR-FTIR (u,cm^{-1}):3159 (NH) str.of sec.amide,3032 Ar(C-H) str.,1678(C=O) str. of amide ,1620 (C=N) str. ^{1}H NMR(500 MHz, DMSO-d6, δ =ppm): 2.34(s, 3H,quinazolinone-CH_3),4.66(s,2H-CH_2-S-),7.55(d,1H,J=8.61,Ar-H),7,65(d,2H,J=8.49,Ar-H) ,7.82 (d,1H,J=8.61 ,Ar-H),8.10(s,1H,Ar-H),8.12(d,2H,J=8.49,Ar-H)12.22(brs,1H,NH),13.21(brs,1H,NH) .^{13}CNMR(125MHz,DMSO-d6):21.38,37.91,120.35,125.78,126.78,128.71,129.60,131.10, 135.07,135.12,137.76,148.96,161.47,166.41, 169.93.

Synthesis of 4-methoxy-N-(5-(((2-methyl-4-oxo-3,4dihydroquinazolin-6-yl)methyl)thio)-1,3,4-thiadiazol -2-yl)benzamide (III): Off-White fluffy powder, yield (45%), m.p (329-332^{0}C), R_f=0.31(n-hexane:ethylacetate :methanol 5:3:2). ATR-FTIR(u,cm^{-1}):3171(NH)str.ofsec.amide,3032 Ar(C-H)str., 1670(C=O) str. of amide ,1604(C=N)str., 1249(C-O) str. of Ar-OCH_3. ^{1}H NMR (500 MHz, DMSO-d6,δ=ppm):2.34(s,3H,quinazolinone-CH_3),3.86(s,3H,O-CH_3),4.65(s,2H-CH_2-S-),7.09 (d,2H, J=8.53,Ar-H),7.55(d,1H,J=8.41,Ar-H),7.82(d,1H,J=8.41,Ar-H), 8.09 (s, 1H, Ar-H), 8.12 (d,2H,J=8.53,Ar-H),12.23(br s,1H,NH) ,12.93 (br s,1H,NH).^{13}CNMR (125 MHz ,DMSO-d6): 21.32, 37.02, 55.97, 113.97,120.31, 123.12,125.77, 126.72,130.51, 132.50, 134.81, 135.11, 154.54, 157.84,160.05,160.18,163.06.

Synthesis of N-(5-(((2-methyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl)thio)-1,3,4-thiadiazol-2-yl)-4-nitrobenzamide(IV): Yellow fluffy powder, yield (51%), m.p (356-358^{0}C) R_f=0.34(n-hexane:ethylacetate: methanol5:3:2). ATR –FTIR(u,cm^{-1}),3159(NH)str.ofsec.amide,3008 Ar(C-H)str. ,1658(C=O) str. of amide, 1601 (C=N) str. ,1558 and1350 asymmetric and symmetric str. of (NO_2) .^{1}H NMR (500MHz,DMSO-d6,δ=ppm):2.34(s,3H,quinazolinone-CH_3),4.67(s,2H-CH_2-S-),7.55 (d,1H,J=8.36,Ar-H),7.83(d,1H,J=8.36Hz,Ar-H),8.13(s,1H,Ar-H),8.30(d,2H, J=8.55 , Ar-H) ,8.37(d,2H,J=8.55,Ar-H),12.21(br s,1H,NH), 13.49 (br s, 1H,NH).^{13}CNMR (125 MHz, DMSO-d6,) : 21.35, 37.91 ,120.33 ,123.66 ,125.76, 126.71,130.64,135.03,135.09 , 136.33,148.12,148.98,149.98,154.34,161.46,165.75,169.93.

Biological evaluation

Maintenance of cell cultures: Cytotoxicity evaluation was performed at the Iraqi Biotech. Research Centre utilizing fetal bovine serum (FBS) Trypsin/EDTA and RPMI 1640 (Capricorn, Germany); cell culture plates and dimethylsulfoxide (DMSO) (Santacruz Biotechnology ,USA); (MTT) stain (Bio-World,USA));microtiter reader (Gennex lab., India); laminar flow hood and CO2 incubator (Cypress Diagnostics, Belgium). Human breast cancer cell line (MCF-7), human lung cancer cell line (A549) and normal human fibroblast (NHF) cell line were grown in RPMI-1640 media supplemented with (FBS,10%), penicillin (100IU/mL), and streptomycin (100 g/mL). The cells were treated with the Trypsin-EDTA and re-seeded at 50% confluence twice a week then incubated at 37 °C [17].

In vitro cytotoxicity MTT assay: MTT assay was done on 96-well plates. Cell lines were seeded at 1 × 10^4cells/well. When a confluent monolayer was obtained, cells were treated with the target compounds (**I-IV**) separately at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µM) using methotrexate as a reference . Viability of the cells was measured after 72hrs. of the treatment by removal of the medium, adding 28 µL of MTT solution (2 mg/mL) and the cells were incubated for 1.5 hrs. at 37 °C. MTT solution was removed, the remaining crystals were solubilized with130 µL of DMSO and incubated for 15 min. at 37 °C with shaking [18]. The absorbency was measured at 492 nm by using microplate reader, the assay procedures were done in triplicate. The rate of inhibition was calculated by using of the following equation: [19] [20]

$$Cytotoxicity\ (\%) = A - B/\ A \times 100$$

Where A denotes the control optical density and B denotes the sample optical density.

Molecular docking stud: ChemBioDraw program was utilized to draw the chemical structures of the ligands and then they have been changed over into a three dimentional structures with ChemBio3D computer program. Energy minimization for the ligands was performed using MM2 job and spared in the form of mol2 files. The crystallized structure of EGFR was downloaded from the protein data bank (PDB ID: 6LUD) [21]. Water molecules, unnecessary ions were deleted from the protein, hydrogen atoms were added and Gasteiger charges were also assigned to mimic the in vivo conditions. The prepared protein then saved in the form of mol2. GOLD algorithm software was used in the docking of the compounds into the EGFR binding site. The reliability of the docking process was validated utilizing the x-ray structure of the co-crystallized ligand (osimertinib) by redocking it into the defined binding site. When the docking run completed, the conformations of the docked ligands were ranked by PLP-fitness scores and the ligand-protein interactions were visualized.

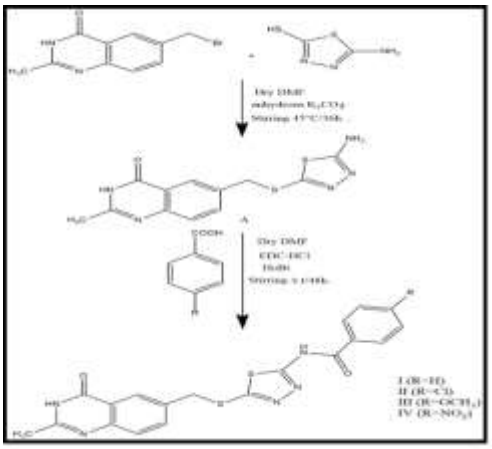
RESULTS AND DISCUSSION

Chemistry: New amide derivatives of 4(3H)-Quinazolinone were synthesized as shown in Scheme 1.

6-(bromomethyl)-2-methyl-4(3H)-quinazolinone and 5-amino-1,3,4-thiadiazole-2-thiol were reacted in dry DMF in the presence of anhydrous potassium carbonate to afford (A) intermediate. Further, the obtained intermediate was reacted with benzoic acid and its derivatives to prepare the target amide derivatives using N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl) as a coupling agent and 1-Hydroxybenzotriazole (HoBt) as a powerful racemization suppressor .Structures of all compounds were confirmed by ATR-

FTIR, ^{1}H-NMR and ^{13}C-NMR. IR spectra of the intermediate A showed the appearance of 2 peaks at 3329 and 3298 cm^{-1} attributed to the asymmetric and symmetric NH_2 stretching vibration of primary amine. The IR spectra of the final compounds characterized by the disappearance of NH_2 stretching peaks and the appearance of medium intensity broad peak in the range 3159-3170 cm^{-1} characteristic of secondary amide NH stretching vibration in addition to the strong peaks of C=O stretching vibration (amide I) band in the range 1658-1678 cm^{-1}. Peaks at 1604–1620 cm^{-1} confirmed the presence of C=N group of the thiadiazole ring. ^{1}HNMR spectra of compound A showed an intense broad singlet peak at 7.27 ppm which signified 2 protons attributed to NH_2 in addition to broad singlet peak of (CONH) of quinazolinone at 12.19 ppm. ^{1}HNMR spectra of the final compounds characterized by the appearance of an additional broad singlet peak at12.93-13.49 ppm due to the aromatic amide (CONH). The methyl group attached to quinazolinone moiety was defined by the appearance of sharp singlet at 2.33–2.34 ppm which signified 3 protons. The aromatic protons were confirmed by multiplet at 7.09–8.37 ppm. In addition to these common groups, the protons of OCH_3 in compound III were identified by the singlet peak at 3.86 ppm having an integration of 3 protons. Characteristic peaks were also found at 161.93-169.93 ppm in the ^{13}CNMR spectra of the final compounds due to the carbonyl group of the aromatic amide (CONH).

In vitro cytotoxicity screening: The cytotoxic activities of the synthesized compounds (I–IV) were evaluated against various human cancer cell lines including MCF-7 (breast) and A549 (lung) in addition to epithelial cells derived from normal human fibroblast (NHF) to investigate the tuomor selectivity exhibited by the tested compounds . All compounds displayed higher cytotoxic activity against the cancer cells than that against the normal cells. There was a continuous increase in the growth inhibition against both breast and lung carcinoma cell lines with increasing the concentration of the tested compounds (figure.2).The IC50 (µM) values of the tested compounds (I-IV) were given in Table 1 . The results indicated that the compounds showed moderate to potent cytotoxic effects against the selected cancer cells. Among the tested compounds, compound I exhibited the highest cytotoxic activity against MCF-7 cell line with IC50 of 7.83 µM values compared to methotrexate IC50 27.32 µM .The results of cytotoxicity against A549 cell line showed that all the tested compounds displayed higher potency than methotrexate with the most potent one is compound III with IC50 6.67 µM in comparison to methotrexate IC50 259.4 µM which could be explained by the potential inhibitory activities of the synthesized compounds against EGFR kinase that overexpressed in lung cancer tissue .



Scheme 1.synthesis of the target compounds (I-IV).

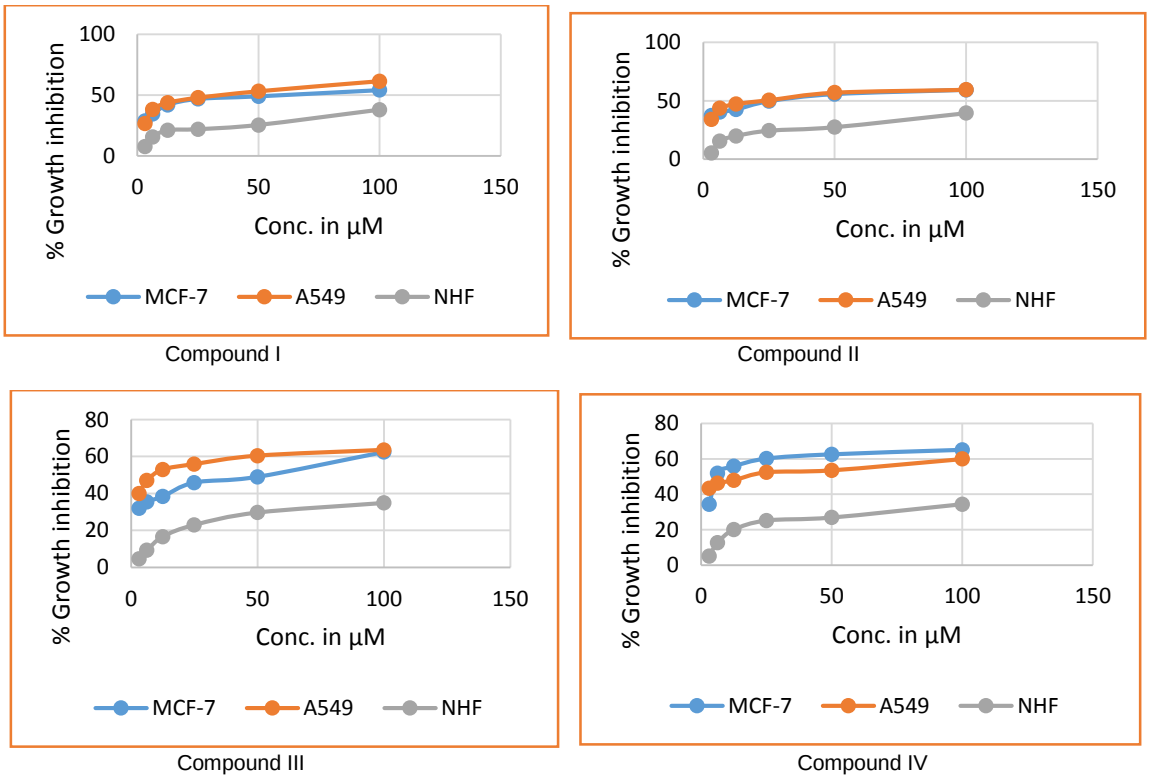
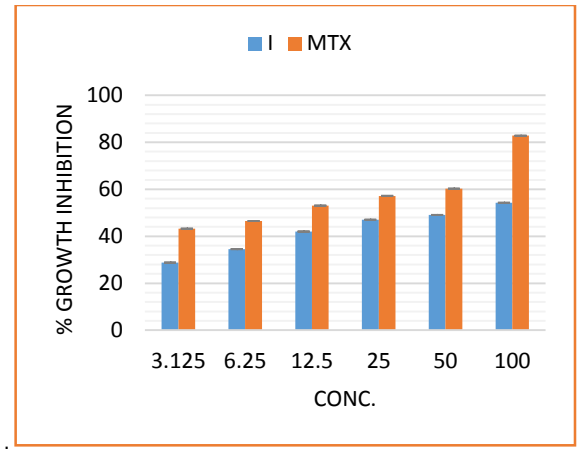
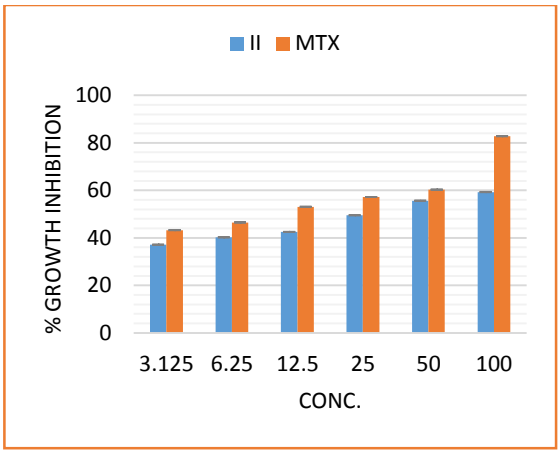


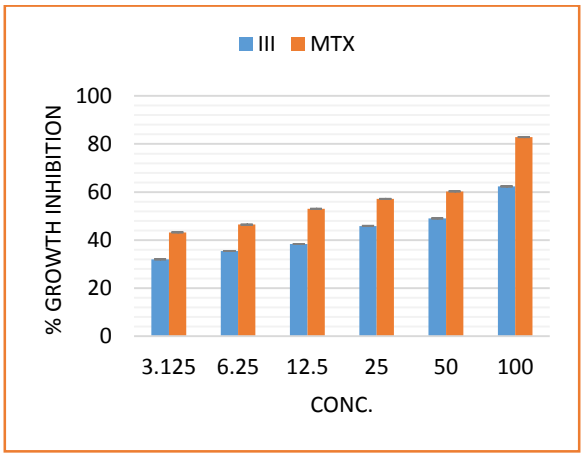
Figure 2 . Concentration versus growth inhibition curve of the tested compounds against breast carcinoma cells (MCF-7) (blue), lung carcinoma cells (A549) (orange) and normal fibroblast cells (NHF) (gray). (values are represented by the mean ± SEM of triplicate measurements).



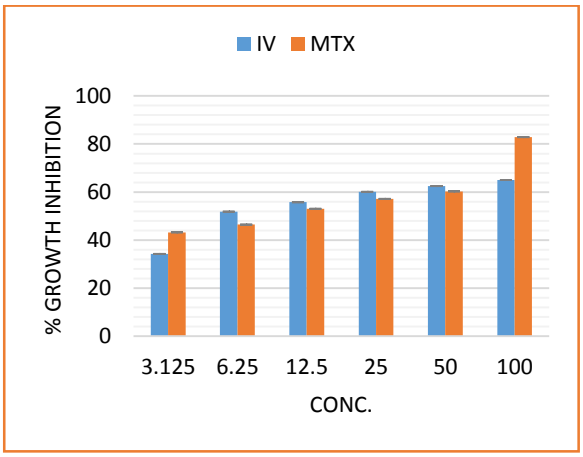
Compound I



Compound II

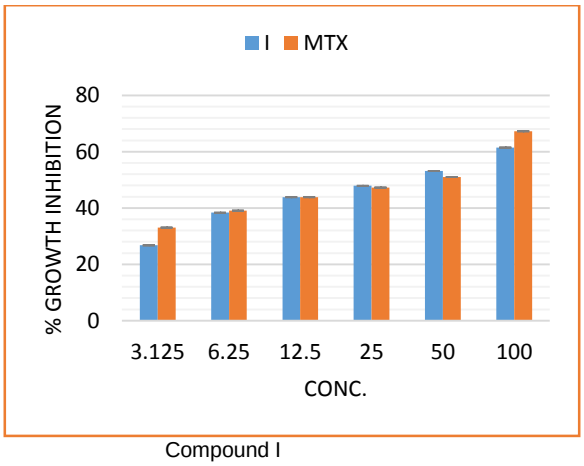


Compound III

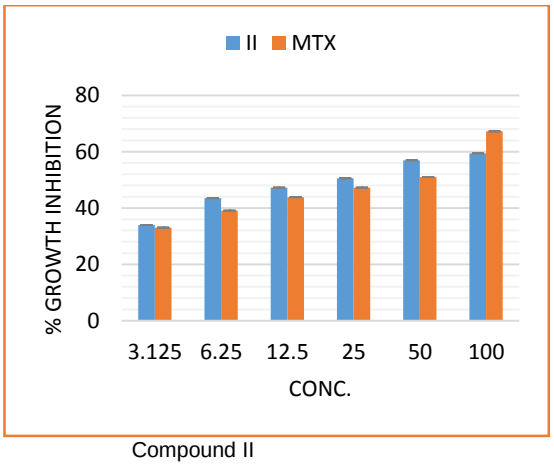


Compound IV

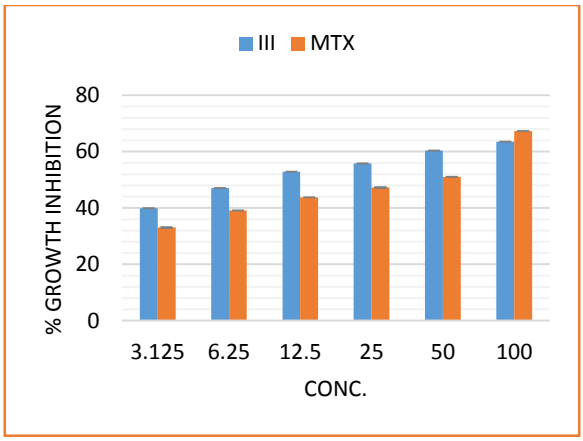
Figure 3. Histogram representing the concentration (μM) versus the growth inhibition of the tested compounds against breast carcinoma cell line (MCF-7). (values are represented by the mean ± SEM of triplicate measurements).



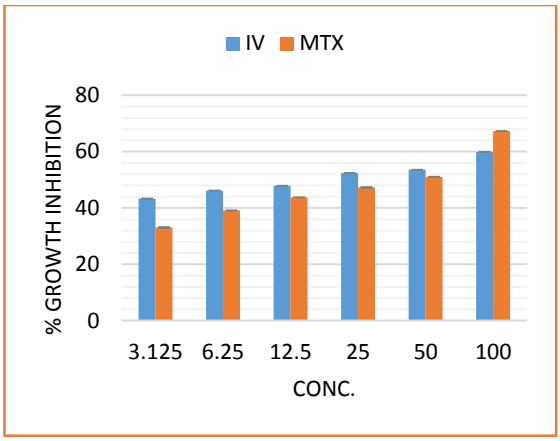
Compound I



Compound II



Compound III



Compound IV

Figure 4. Histogram represnting the concentration (µM) vesus the growth inhibition of the tested compounds against lung carcinoma cell line (A549). (values are represented by the mean ± SEM of triplicate measurements).

Table 1: cytotoxic activity (IC50) of compounds I–IV against two cancer cell lines.

Compound	IC50 (µM)	
	MCF-7 cell line	A549 cell line
I	7.83	9.63
II	29.69	7.20
III	95.37	6.67
IV	17.48	44.29
MTX	27.32	259.4

Structure Activity Relationship (SAR): The unsubstituted derivative (I) showed higher antitumor activity than the P-substituted derivatives on breast cancer cell line (MCF-7). The introduction of an electron withdrawal substituent in para position was shown to increase the antitumor activity against MCF-7 cells when compared with electron donating group, compounds II and IV with the electron withdrawing groups (Cl and NO_2) respectively exhibited higher antitumor activity against MCF-7 cells than compound III with the electron donating methoxy group . On the other hand, introduction of the electron donating methoxy group in the para-position of compound III showed an improvement in the cytotoxic activity against the lung cancer cell line (A549) in comparison to the electron withdrawal substituents .

Molecular docking study: The docking study was carried out on compounds (I-IV) as the ligands, where the structure of complex of EGFR (PDB ID: 6LUD) and osimertinib was chosen as the docking pattern. The docking results displayed that all compounds exhibited good binding interactions with PLP fitness scores in the range (61.79 - 72.51). Docking studies showed that all the compounds have more binding interactions than osimertinib with the EGFR binding site. the most potent compounds against MCF-7 cell line (**I** and **IV**) exhibited hydrogen bonding interactions between N atom of the thiadiazole ring in each compound and MET793 residue in the active site of EGFR with a bond lengths of 3.066$A^0$and 2.942A^0 respectively. This confirms that incorporation of 1,3,4-thiadiazole moiety enhance the binding interactions with EGFR target protein and improve the activity against (MCF-7) cells by inhibiting EGFR signalling pathway**.** Compound (**III**) , the most potent one against (A549) cells showed different binding pattern at EGFR binding site in which the hydrogen bonding interactions involved quinazolinone NH and ARG841 residue with 3.054 A^0 bond length and aromatic hydrogen bonding interaction with LEU792 residue having 2.876 A^0 distance (figure 5).

In silico ADME Prediction: The online ADME software was used to predict the physicochemical properties as well as the virtual prediction of (ADME) features and drug-like properties of the designed compounds to identify the compounds to be drug candidates and exclude others that display unfavourable ADME features in the next stages of drug development. The physicochemical properties of drugs play an important role in their biological activity . Partition coefficient (Clog P) is one of these properties which give prediction about the pattern of drug movement inside the body. Clog P values of the target compounds were less than five (table 3) . The target compounds were also showed acceptable bioavailability scores (0.55) and were also seemed to be not substrates for P-glycoprotein (multidrug resistance protein). The synthesized compounds displayed no clear violation of lipinski rule of five .

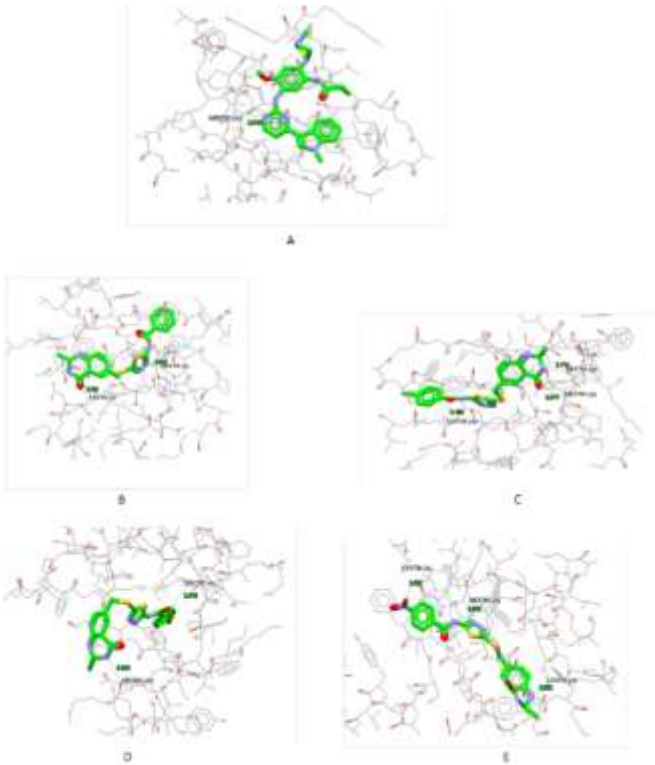


Figure 5: Interaction of osimertinib (A), compound I (B),compound II (C) compound III and (D), compound IV (E) with the amino acid residues of EGFR (pdb:6LUD).

Table 2: Drug Likeness and Lipinski rule descriptors of the target compounds.

Comp.	M.wt	no.H-bond acceptors	no.H-bond donors	TPSA	C log p	Lipiniski violation	P_gp substrate	Bioavailability score
I	409.48	5	2	154.17	2.97	0	NO	0.55
II	443.93	5	2	154.17	3.52	0	NO	0.55
III	439.51	6	2	163.4	3.01	0	NO	0.55
IV	454.48	7	2	199.99	2.24	0	NO	0.55

CONCLUSION

New amide derivatives of quinazolin-4(3H)-ones have been synthesized and their antitumor activity was evaluated against two cancer cell lines, breast (MCF-7) and lung (A549). Among the compounds that tested on MCF-7 cell line , the most remarkably was compound I which exhibited the highest cytotoxic activity (IC50 7.83 µM) having more than three folds greater activity than that of methotrexate (IC50 27.32µM). All compounds showed superior cytotoxic activity than methotrexate against A549 cell line, the most potent one is compound III with IC50 value of 6.67 µM . The low cytotoxicity of the tested compounds against the normal cells gives a hope of the ability of these compounds to target the cancer cells with a higher degree than the healthy cells .Molecular docking studies showed that the incorporation of 1,3,4-thiadiazole moiety into quinazolin-4(3H)-ones core could enhance EGFR inhibition . The obtained significant cytotoxic activities of these new hybrid molecules can be considered as a useful model for the future design and further modifications to obtain compounds with potent antitumor effects.

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