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Synthesis, and Anti-Tumor Evaluation of Some New *Flurbiprofen* Derivatives against MCF-7 and WRL-68 Cell Lines

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ABSTRACT

A new series of flurbiprofen derivatives containing thiosemicarbazide moiety (**3-7**) was synthesized from flurbiprofen as parent nucleus by esterification, hydrazide formation, and heating with different aryl isothiocyanate substituents, respectively. Flurbiprofen was also treated with thiosemicarbazide in the presence $POCl_3$ as a catalyst, to produce 1,3,4 -thiadiazole -2-amine (**8**). Treatment of (**8**) with different aryl isothiocyanates produced thiourea derivatives (**9-12**). Also, the reaction of (**8**) with different benzoyl chloride substituents produced benzamide compounds (**13-15**). Eventually , treatment of (**8**) with ethyl acetoacetate (EAA) produced [1,3,4]thiadiazolo[3,2-a]pyrimidin-7-one (**16**) .The new compounds were characterized by spectroscopic techniques :FTIR, ^{1}HNMR, and CHNS analysis. A molecular docking study for the synthesized compounds (**3-16**), against the *Vascular Endothelial Growth Factor receptor (VEGFR-2)* was applied, and it indicated that compounds **4,7,13,** and **15**, exhibited the optimum binding energy of -6.77,-6.12,-6.68,-6.43 kcal/mol, respectively. Target compounds were also assessed for their *in vitro* anticancer effects in a cell-line study. All of the compounds tested showed the most plausible anticancer activity, compared to a positive control (sorafenib), using *in vitro* MTT cytotoxic assay, against human breast tumor (MCF-7), and normal WRL-68 cell line. The *in vitro* results revealed that compounds **4,5,10,11,13**, and **15** exhibited the highest inhibitory activity at their IC_{50} concentrations, against MCF-7 cell lines, as follows (122.7,113.9,95,7. 109.1,40.32 and 112.29µg/mL, respectively. While their cytotoxic effect against normal WRL-68 cell line at their IC_{50} concentrations, as follow 210.2,181.3,151.7,278.7,80.28, and 236µg/mL, respectively. Therefore, such compounds were considered more selective toward MCF-7 than normal WRL-68, and their selectivity index (SI): 1.71,1.59,1.59, 2.55,1.99 , and 2.10, respectively. Among the synthesized compounds, the compound **15** was chosen to screen its effect *in vitro* through multi-parameter cytotoxic assay against MCF-7 breast cancer implemented in High Content Screening (ArrayScan XTI, Thermo Scientific),which could be taken in consideration as a starting point for the development of new anticancer drugs.

Keywords: Thiosemicarbazide, anti-tumor activity, thiourea, aryl isothiocyanate, high content screening.

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INTRODUCTION

New anti-cancer drugs that have already been developed and discovered are a significant strategy for the treatment of malignant tumors, an incredibly touching field for medicinal chemists to find compelling yet safer chemotherapeutic drugs that focus on different biological mechanisms responsible for the development of various cancers (Magalhaes *et al*., 2018). Among these targets, angiogenesis is one of the basic processes affecting the growth and development of cancerous cells (Nishida *et al*., 2006). Angiogenesis is a complex process managed by various growth factors and cytokines. Among such indicators, vascular endothelial growth factor (VEGF) is one of the most vital angiogenic factors' involved cancer development. VEGF" has two receptors, receptor 1 (VEGFR-1) and receptor 2 (VEGFR-2) (Sadeghinia *et al*., 2019). VEGFR-1 and VEGFR-2 are becoming strategic goals for the improvement of anticancer drugs. Inhibition of the signaling pathway of the VEGFR-2 has an important anti- angiogenic impact on tumors, hence the Food and Drug Administration (FDA) had also approved the small molecule VEGFR-2 kinase inhibitors sorafenib, sunitinib, and pazopanib for the management of serious renal cell carcinoma (Sadeghinia *et al*., 2019).

In this regard, thiosemicarbazide, 1,3,4-thiadiazol-2-amine, thiourea, benzamide, and [1,3,4]thiadiazole[3,2-a]pyrimidine-7-one components form significant category of compounds with a broad array of applications, one of which is anticancer. Thiosemicarbazide has an anticancer activity related to its ability to suppress the enzyme ribonucleotide reductase, as seen with potent anticancer medications, like triapine and methisazone (Arora *et al*., 2014). According to the previous studies 1,3,4-thiadiazol-2-amine has potent VEGFR-2 kinase inhibitory effects (Tripathy *et al*., 2007). 1,3,4-Thiadiazole is a unique five membered rings system that has acquired noticeable quality by exploring wide spectrum of biological activity, due to the existence of the -N=C–S component. Previous literature has already shown that 1,3,4-thiadiazole-derivatives possess diverse biological activities, such as , antibacterial (Jain *et al*., 2013, Mathew *et al*., 2007), anti-hepatitis B infections (Kushwaha *et al*., 2012), anti-tubercular (Kushwaha *et al*.,2012; Foroumadi *et al*.,2005), anti-leishmanial (Siddiqui *et al*.,2009), pain relieving, and anti-inflammatory (Kushwaha *et al*., 2012; Mathew *et al*., 2007), anti-cancer (Gomha *et al* 2015; Gomha *et al.,*2015; Kumar *et al*., 2006), anticonvulsants (Siddiqui *et al*., 2009; Sharma *et al*., 2013), central nervous system (CNS) depressants (Siddiqui *et al.*, 2009), anti-oxidant (Kushwaha *et al*., 2012), diuretics, and antihypertensive activities (Kushwaha *et al*., 2012; Bhattacharya *et al*., 2005). Recently, some moieties containing 1,3,4-thiadiazoles were investigated for their potential anti-tumor and anti-microbial effects through the inhibition of dihydrofolate reductase (DHFR) (Riyadh *et al*., 2018). Thiourea induces apoptosis in cancer cell lines (Huangab *et al.*, 2017). Benzamide derivative showed potential cytotoxic action against colon HCT-116 cells (Chan *et al*., 2018). The chemistry and biological research on pyrimidines and their analogs have drawn extensive attention because, in addition to their impressive anticancer activities, such a ring structure is the primary skeleton in alkaloids and nucleic bases (Yousif *et al.,* 2017).

The current research aimed to synthesizes new flurbiprofen derivatives with interesting anticancer activity through the predicted VEGFR-2 kinase inhibitors from docking study, these derivatives containing 1,3,4-thiadiazol-2-amine, thiourea, benzamide, thiosemicarbazide, and [1,3,4]thiadiazolo[3,2-a]pyrimidin-7-one moieties, were synthesized and evaluated *in vitro* for their anticancer activities against MCF-7 and WRL-68 cell lines. Since VEGFR was over expressed in MCF-7cell, therefore these cell lines were chosen in this study (Dore-Savard *et al*., 2016).

MATERIAL AND METHODS

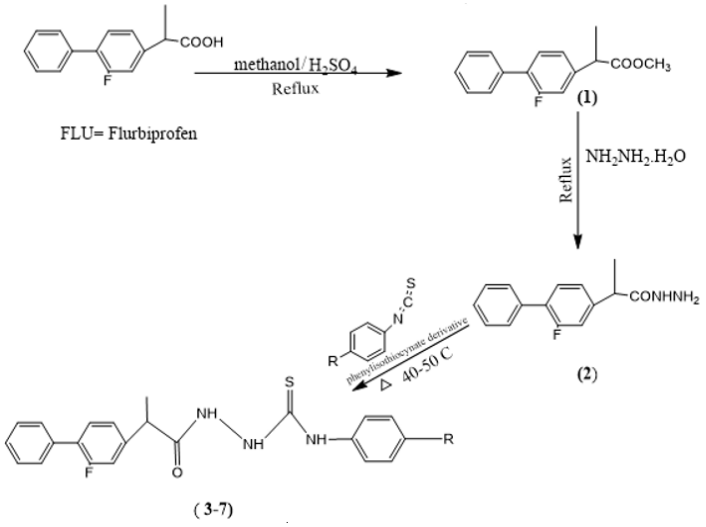
R-Flurbiprofen was purchased from Sigma-Aldrich."FTIR" spectra were recorded using spectrophotometer on KBr" disk, ($v = cm^{-1}$). CHNS micro-analysis was carried out using Euro EA3000 elemental analyzer, and ^{1}HNMR spectra were recorded on Varian Inova Spectrometer, 500MHz utilizing (TMS) as an internal standard. The chemical 'shifts were expressed as (δ=ppm), $DMSO_{d6,}$ $CDCl_3$ and CD_3OD were utilized as solvents.

Chemical synthesis

The synthesis of new derivatives (Figures1 and 2).

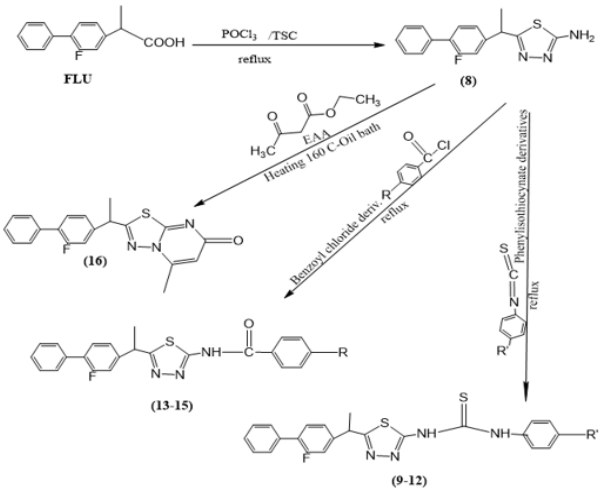
Synthesis of methyl 2-(2-fluoro-[1,1'-biphenyl]-4-yl) propanoate ... (1) (Telehoiu *et al.*, 2020; Al-Wabli *et al.*, 2017)

Flurbiprofen (10mmol, 2.44g) was dissolved in 30mL of abs. MeOH, then few drops of conc. H_2SO_4 were added , the resulting mixture was refluxed for 3h, and monitored with TLC.



Compound	R
3	H
4	Br
5	Cl
6	OCH_3
7	NO_2

Figure1. Synthesis of compounds (1-7).



Compound	R'
9	H
10	Br
11	Cl
12	NO_2

Compound	R
13	H
14	OCH_3
15	NO_2

Figure 2. Synthesis of compounds (8-16).

Then cooled, and neutralized by $NaHCO_3$ (5%). The product (oil) was extracted twice with diethyl ether, and then, the extracted solvent was evaporated to form compound (**1**).

White powder, yield 78%, m.p: 98-101^{0}C, $R_f$value = 0.59, IR (KBr disc, v= cm^{-1}): 3068 and 3032 (Ar-CH) str, 2943 and 2881(CH) str of *aliph*. CH & CH_3, 1890, 1915, 1952 (Aromatic overtone/ combination bands), 1736 (C=O) ester str, 1171. (C-O-C) *asym.*str, 1070 (C-O-C) OCH_3 *sym* str, 1577, 1560 and 1483 Ar(C=C) str.

CHNS analysis: Calcd. for ($C_{16}H_{15}FO_2$): C: 74.4, H: 5.85. Observed: C: 74.20, H: 5.32, ^{1}HNMR (500MHz, $CDCl_3$, δ=ppm); 7.26-7.03 (8H, m, Ar-H); 3.93 (1H, q, CH-*aliph.*); 3.69 (3H, s, OCH_3); 1.53 (3H, d, CH_3).

Synthesis of 2-(2-fluoro-[1,1'-biphenyl]-4-yl) propanehydrazide ... (2) (Parmar *et al.,* 2011; Koopaeia *et al.,* 2013)

(5.0mmol 1.3g) of compound**(1)** was dissolved in (20) mL of abs. EtOH, then (10.0 mmol, 0.33ml) of $NH_2NH_2.H_2O$ was added slowly with stirring for 2h at RT, then the mixture refluxed for 6h. The solvent was evaporated, the mixture was allowed to cool, and the precipitate was already washed with ice-cold water several times, and recrystallized from MeOH.

White precipitate, yield 82%, m.p: 77-79°C, R_f.value= 0.88. IR (KBr disc, (v=cm^{-1}): The Shoulder at 3210 and peaks at 3309 , and 3178 (NH) *prim*. amine str, 3030 Ar (CH) str, 2978, 2933 and 2879 (CH) str of *aliph*.CH & CH_3, 1900, and1961 (Aromatic overtone/combination bands), 1635 (C=O) of amide hydrazide str , 1523, 1483 and 1415 Ar(C=C) str. CHNS analysis: Calcd. for ($C_{15}H_{15}FN_2O$) : C : 69.75,H: 5.85, N: 10.85. Observed: C: 69.25, H: 5.36, N: 10.72.

^{1}HNMR (500MHz, $CDCl_3$, δ=ppm) 7.28-7.02 (8H, m, Ar-H); 3.87 (2H, *br*s, NH_2); 3.55 (1H, q, CH-*aliph*.); 1.55 (3H, d, CH_3).

General method for synthesis of the compounds (3-7)…… (Hui *et al*., 1994; Al-Saad *et al*., 2019)

(2.0 mmol, 0.517g) of compound **(2)** was dissolved in (20mL) abs. MeOH, then (2.0 mmol) of each different substituted aryl-isothiocyanates: (0.24mL) phenylisothiocyanate; (0.43g) *p*.bromo phenylisothiocaynate; (0.338g) *p*.chloro phenyl-isothiocynate; (0.28mL) *p.* methoxy phenylisothio-cyanate, and (0.360g) *p.*nitro phenylisothiocaynate, each was added separately with stirring. The temperature of the reaction was kept at 40-50°C. The precipitate was filtrated, then washed with EtOAc, and recrystallized from EtOH.

Compound (3): 2-(2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoyl)-N-phenylhydrazine-1-carbothioamide.

White precipitate, yield 72%, m.p: 163-165°C, R_f.value = 0.37, IR (KBr disc,v= cm^{-1}): 3346, 3246 and 3136 (NH) str of (1stamide, 2nd amine and NH adjacent to thiocarbonyl), 3060 and 3032 Ar (CH) str, 2966 , and 2879 (CH) str of *aliph*. CH & CH_3, 1944 (Aromatic overtone), 1676 (C=O) of amide hydrazide str, 1595, 1537, 1508, and 1448 Ar(C=C) str, 1190 (C=S) str. CHNS analysis: Calcd. for ($C_{22}H_{20}FN_3OS$) C: 67.16, H: 5.12, N: 10.68, S: 8.15. Observed: C: 67.86, H: 4.85, N: 11.00, S: 8.55. ,^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 10.15 (1H,s, NH); 9.51 (1H,s, NH); 7.70-7.05 (13H,m, Ar-H); 3.75 (1H,d, CH-*aliph*.); 1.40 (3H,d, CH_3).

Compound (4): N-(4-bromophenyl)-2-(2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoyl)hydrazine-1-carbothioamide.

White precipitate, yield 65%, m.p:179-181^{0}C, R_f. value = 0.48, IR (KBr disc, v= cm^{-1}): 3239 , 3184 *br* (NH) str of (1st amide, 2nd amine and NH adjacent to thiocarbonyl), 2978 (CH) str of *aliph*.CH & CH_3, 1886 (Aromatic overtone),1685 (C=O) str of 1stamide, 1593,1545,and 1487 Ar(C=C) str, 1176 (C=S)str, 766 (C-Br) str, 823 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{22}H_{19}BrFN_3OS$) C: 55.94, H: 4.05, N:8.90, S: 6.79 . Observed: C: 54.94, H: 3.31, N: 8.64, S: 6.74. ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 10.15 (1H,s, NH); 9.69 (1H, s, NH); 7.51(2H, d, 2Ar-H); 7.48 (2H,d, 2Ar-H); 7.46-7.27 (m,8H,Ar-H); 3.76 (1H, q, CH-*aliph*.); 1.43 (3H, d, CH_3).

Compound (5): N-(4-chlorophenyl)-2-(2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoyl) hydrazine-1-carbothioamide.

Pale white precipitate, yield 45%, m.p: 190-192^{0}C, R_f. value = 0.27, IR (KBr disk v =cm^{-1}): Shoulder at 3334, 3238, 3182 *br* (NH) str of (1st amide, 2nd amine and NH adjacent to thiocarbonyl), 2978, and 2883 (CH) str of *aliph*. CH & CH_3, 1866 (Aromatic overtone), 1685 (C=O) str of 1st amide; 1593, 1545, 1487 and 1415 Ar-(C=C) str, 1178 (C=S) str, 1072 (C-Cl) str, 823 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{22}H_{19}ClFN_3OS$) C: 61.75, H: 4.48, N: 9.82, S: 7.49. Observed: C: 61.32; H: 4.96, N: 8.94, S: 6.81, ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 10.16 (1H, s, NH); 9.68 (1H, s, NH);7.51(d,2H,Ar-H); 7.46 (2H, d, Ar-H); 7.45-7.27 (8H, m, Ar-H); 3.76(1H,q, CH-*aliph*.); 1.43 (3H, d, CH_3).

Compound (6): 2-(2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoyl)-N-(4-methoxyphenyl) hydrazine-1-carbothioamide.

Pink precipitate, yield 62%, m p:215-218°C, R_f. value = 0.38, IR (KBr disc,v = cm^{-1}): 3336,3309 and 3138 (NH) str of (1st amide, 2nd amine and NH adjacent to thiocarbonyl),3066,3033 Ar(CH) str, 2964, and 2839 (CH) str of *aliph.* CH & CH_3, 1684 (C=O) str of 1st amide,1543, 1510 and 1450 Ar(C=C) str, 1250 *asym* (C-O) str, 1176 (C=S) str, 1030 *sym* (C-O) str, 837 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{23}H_{22}FN_3O_2S$) C: 65.23, H: 5.24, N: 9.92, S: 7.57. Observed: C: 64.90, H: 4.82, N: 9.36, S: 7.42., ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 10.11 (1H, s, NH); 9.46 (1H, s, NH); 7.50-7.23 (10H, m, Ar-H); 6.88 (2H, d, 2Ar-H); 3.75 (1H, q, CH-*aliph*.); 3.69 (3H, s, OCH_3); 1.43 (3H, d, CH_3).

Compound (7): 2-(2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoyl)-N-(4-nitrophenyl)hydrazine-1-carbothioamide.

White precipitate, yield 48 %, m.p: 192-195°C, R_f value = 0.26, IR (KBr disc, v = cm^{-1}): 3298 overlap (NH) str of (1st amide and 2nd amine); 3155

(NH) str adjacent to thiocarbonyl, 3055 Ar(CH) str; 2978, 2939 and 2833 (CH) str of *aliph*. CH & CH_3, 1685 (C=O) amide str, 1618 and 1574 Ar (C=C) str, 1184 (C=S) str, 1504 (NO) *asym.* str, 1333 (NO) *sym* str, 853 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{22}H_{19}FN_4O_3S$): C: 60.26, H : 4.37, N: 12.78, S: 7.37. Observed: C: 59.17, H:3.98 ,N:12.42, S:7.54. ^{1}HNMR (500MHz, DMSO$_{d6}$, δ=ppm); 10.03 (1H, s, NH); 8.19 (2H, d, Ar-H); 7.86(d,2H,Ar-H); 7.52-7.27 (8H, m, Ar-H); 3.79 (1H, q, CH-*aliph)*; 1.44 (3H,d, CH_3).

Synthesis of 5-(1-(2-fluoro-[1,1'-bi phenyl]-4-yl) ethyl)-1,3,4-thiadiazol-2-amine….. (8) (Tomi *et al*.,2014; Nahi *et al.,*2019)

A mixture of flurbiprofen (5.0mmol, 1.22g), thiosemicarbazide (5.0mmol, 0.455g,) and $POCl_3$ (5mL) were refluxed for 4h. Then the mixture was cooled and ice water (25mL) was added gradually and the mixture of reaction was refluxed for further 3h, then cooled and filtered. Neutralization of the filtrate with KOH solution (10%) and the precipitate was also filtered, and re-crystallized from MeOH.

Brown precipitate, yield 62%., m.p 216-219°C, R_f .value= 0.38, FTIR (KBr, v= cm^{-1}): 3280 and 3118 str for *prim*. (NH_2), 3035 (Ar-CH) str, 2976 , 2933 and 2875 (CH) str. of *aliph*.-CH & CH_3,1952, 1892 (Aromatic overtone/combination bands), 1622,1608 (Ar-C=N)str, 1514,1485,1415 (Ar-C=C) str, 1130 (N-N)str, 766 and 725 C-S str. CHNS analysis: Calcd. for ($C_{16}H_{14}FN_3S$) C: 64.19 , H:4.71 , N: 14.04, S:10.71. Observed: C:63.85, H: 4.81, N: 13.80, S: 10.45. ^{1}H- NMR (500MHz,CD_3OD,, δ=ppm); 7.46-7.07 (8H,m, Ar-H); 4.45 (1H,q, CH-*aliph*.); NH_2(masked by CD_3OD-solvent); 1.68 (3H,d,CH_3).

General method for synthesis of 1-(4-chlorophenyl)-3-((5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-ylthiourea derivatives…(9-12) (Rahman *et al*., 2014)

A mixture of compound (**8**) (0.375g, 1.25 mmol), and (1.25 mmol) of different substituted aryl isothiocyanates: phenyl isothiocyanate (0.15mL), *p*.bromo phenyl isothiocyanate (0.276g), *p*.chloro phenyl isothiocyanate (0.212g), *p*.nitro phenyl isothiocyanate (0.225g), each was added separately in acetone (20mL), and heated under reflux for 24h. The reaction mixture was then evaporated, left to cool, and the solid formed was already filtered, washed with acetone, dried and recrystallized from EtOAc–MeOH to yield the desired compound.

Compound 9 : 1-((5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)-3-phenylthiourea .

White precipitate, yield 49%, m.p:193-196 °C, R_f.value = 0.46, IR (KBr disc, v = cm^{-1}): 3456, 3323, 3261 and 3197 (NH) str, 3089 and 3028 (CH) str of aromatic CH, 2979, 2877 (CH) str, of *aliph*. CH and CH_3, 1597, 1550, 1489 and1435 Ar(C=C) str, and 1657Ar(C=N)str, 1234 (C=S) str, 1128 (N-N)str, 756 C-S str. CHNS analysis: Calcd. for ($C_{23}H_{19}FN_4S_2$) C: 63.57, H: 4.41 , N:12.89 , S:14.76 . Observed: C: 64.01, H: 3.98, N: 12.12, S: 13.93. ^{1}HNMR (500MHz, $(CD_3)_2CO$, δ=ppm); 7.67-7.09 (13H, m, Ar-H); 4.40 (1H, q, CH-*aliph*.); 1.42 (3H, d, CH_3).

Compound 10 : 1-(4-bromophenyl)-3-((5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)thiourea

Pale white precipitate, yield 52%, m.p:202-205°C, R_f.value = 0.47, IR (KBr disc, v = cm^{-1}): shoulder at 3200, 3128 for (NH)str, 3060 (Ar-CH) str , 2987, 2935, (CH) str of *aliph.* CH and CH_3 , 1512, 1481 and 1414 Ar(C=C), and 1601& 1624 Ar(C=N) str, 1263 (C=S) str, 1136 (N-N)str, 739 (C-S) str, 768 (C-Br) str , 854 and 833 (Aromatic sp^2 CH) bend. of *para* position . CHNS analysis: Calcd. for ($C_{23}H_{18}BrFN_4S_2$) C: 53.80 ,H:3.53, N:10.91 ,S:12.49 . Observed: C: 54.86, H:3.27 ,N:10.44,S:13.02. ^{1}HNMR (500MHz, DMSO$_{d6}$, δ=ppm); 10.38 (1H, s, NH); 7.73 (2H, d, Ar-H); 7.55-7.31 (10H, m, Ar-H); 4.55 (1H, q, CH-*aliph*.); 1.68 (3H, d, CH_3).

Compound 11 : 1-(4-chlorophenyl)-3-((5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)thiourea

Pale white precipitate , yield 44%, m.p: 207-209°C, R_f.value = 0.47, IR (KBr disc, v = cm^{-1}): 3444,3323,3261 and 3197 str for (NH),3076 3026 (Ar-CH) str, 2978, 2929and 2883 (CH) str of *aliph.* CH and CH_3, 1658 Ar(C=N) str, 1597, 1545, 1489 and 1433 Ar(C=C) str, 1230 (C=S) str, 1124 (N-N)str, 768 (C-S)str, 1099(C-Cl)str, 831 (Aromatic sp^2 C-H) bend. of *para* position. CHNS analysis: Calcd. for $C_{23}H_{18}ClFN_4S_2$) C: 58.90 ,H:3.87 ,N:11.59 , S:13.67. Observed: C:58.84 ,H: 4.11, N:11.43, S:12.95. ^{1}HNMR (500MHz, DMSO$_{d6}$, δ=ppm); 10.38 (1H, s, NH); 7.72 (2H, d, Ar-H); 7.53-7.32 (10H, m, Ar-H); 4.55 (1H, q, CH-*aliph.*); 1.68 (3H, d, CH_3).

Compound 12 : 1-((5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)-3-(4-nitrophenyl)thiourea

White precipitate, yield 44%, m.p: 207-209°C, R_f.value = 0.65, IR (KBr disc, v = cm^{-1}): 3435, 3236 str of (NH), 3037 (Ar-CH) str, 2978, 2935and 2875 (CH) str of *aliph.* CH and CH_3 , 1628, 1592 Ar(C=N) str, 1485 and 1446 Ar(C=C) str, 1275 (C=S) str, 1223 (N-N)str, 762 (C-S)str, 1535. (NO) *asym*. str, 1327 (NO) *sym*. str, 829 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{23}H_{18}FN_5O_2S_2$) C: 57.61, H: 3.78, N:14.60, S:13.37. Observed: C 58.01, H:3.91, N:14.12, S:12.89. ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 10.86 (1H, s, NH); 8.15 (2H, d, Ar-H); 8.05 (2H, m, Ar-H); 7.55-7.29 (8H, m, Ar-H); 4.59 (1H, q, CH-*aliph*.); 1.70 (3H, d, CH_3).

General method for synthesis of the compounds (13-15) (Issaca *et al.,* 2012)

(0.374g, 1.25mmol) of the compound (**8**) was dissolved in (20mL) dry benzene containing triethylamine (3drops) as a catalyst, 1.25mmol of different substituted benzoyl chlorides: benzoyl chloride (0.16mL), *p*-methoxy benzoyl chloride (0.17mL), *p*-nitro benzoyl chloride (0.232g), each was added separately, drop wise to an ice-cooled, stirred solution. The mixture was stirred at RT, then refluxed, until reaching the end after 4h, consequently, cooled at RT. The separated solid was filtered off, and recrystallized from chloroform to produce compounds **(13-15).**

Compound 13: N-(5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)benzamide

White precipitate, yield 67%, m.p: 185-187 °C, R_f. value = 0.56, IR (KBr disc, v = cm^{-1}): Shoulder at 3240, and peak at 3155 (NH) str of amide, 3053 and 3035 Ar (CH) str, 2976 and 2935 (CH) str of *aliph*. CH & CH_3, 1670 (C=O) of amide str, 1622 Ar(C=N) str, 1593., 1537, and 1485,Ar(C=C) str, 1228 (N-N)str, 744 (C-S)str, CHNS analysis: Calcd. for ($C_{23}H_{18}FN_3OS$) C: 68.47, H:4.50, N:10.41,S:7.95. Observed: C 67.97, H: 4.86, N:10.84, S:7.67. ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 12.98 (1H, s, NH); 8.08 (2H, d, Ar-H), 7.63-7.37 (11 H, m, Ar-H); 4.73 (1H, q, CH-*aliph*.); 1.77 (3H, d, CH_3).

Compound (14): N-(5-(1-(2-fluoro-[1,1'-biphenyl] -4-yl)ethyl)-1,3,4-thiadiazol-2-yl)-4-methoxybenzamide.

White precipitate, yield 70%,m.p:192-195°C, R_f = 0.68, IR (KBr disc, v = cm^{-1}): Shoulder at 3220 and peak 3163 (NH) str of amide, 3057,3035 Ar (CH) str, 2976, 2937 and 2800 (CH) str of *aliph.* CH & CH_3,1664 (C=O) of amide str, 1612 Ar(C=N) str, 1533, 1510 and 1480 Ar(C=C) str, 1261 *asym.* (C-O) str, 1184 (C=S)str, 1041 (C-O) *sym.* str ,1130 (N-N)str, 742 (C-S)str, 837 (Aromatic sp^2 CH) bend. of *para* position. CHNS analysis: Calcd. for ($C_{24}H_{20}FN_3O_2S$) C: 66.50, H: 4.65,N:9.69 ,S:7.40. Observed: C 65.90, H: 4.88, N: 9.59, S:7.64. ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 12.89 (1H, s, NH); 7.99 (2H, d, Ar-H); 7.54-7.31 (10 H, m, Ar-H); 4.72 (1H, q, CH-*aliph*.); 2.36 (3H, s, OCH_3); 1.76 (3H, d, CH_3).

Compound (15): N-(5-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-1,3,4-thiadiazol-2-yl)-4-nitrobenzamide

White precipitate, yield 65%, m p 197-200 °C , R_f. value = 0.47, IR (KBr disc, v= cm^{-1}): 3172, (NH) str of amide, 3118 ,3055 Ar (CH) str, 2976 , 2935 and 2870 (CH) str of *aliph*. CH & CH_3, 1678 (C=O) of amide str, 1604 Ar(C=N) str, 1483 Ar(C=C) str, and 1531 (NO) *asym* str, 1344 (NO) *sym* str, 1130 (N-N)str, 704 (C-S) str, 845 (Aromatic sp^2 CH) bend of *para* position. CHNS analysis: Calcd. for ($C_{23}H_{17}FN_4O_3S$) C :61.60 ,H:3.82, N:12.49, S:7.15. Observed: C 61.32, H:3.69, N:11.95, S:7.09. ^{1}HNMR (500MHz, $DMSO_{d6}$, δ=ppm); 8.34 (2H, d, Ar-H)); 8.27(d,2H,Ar-H); 7.53-7.31 (8 H, m, Ar-H); 4.73 (1H, q, CH-*aliph*.); 1.76 (3H,d, CH_3).

Synthesis of 2-(1-(2-fluoro-[1,1'-biphenyl]-4-yl) ethyl)-5-methyl-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-7-one ……(16) (Rahman *et al., 2014*)

(1.25mmol, 0.374g) of compound (**8**) and ethyl acetoacetate (EAA) (1.25 mmol, 0.162g,) were heated at 160°C in oil bath for 6h, upon completion of the reaction, the mixture was therefore cooled at RT. The separated solid was filtered off and recrystallized from chloroform.

White powder, yield 78%, m. p 240-242°C, R_f. value = 0.68, IR (KBr disc, v = cm^{-1}): 3075,3060 Ar (CH) str, 2914 and 2804 (CH) str of *aliph.* CH & CH_3, 1711cm (C=O) str of amide, 1624 Ar(C=N)str, 1572, 1481 Ar(C=C) str, and 1161 (N-N)str,768 and 739 (C-S)str.

CHNS analysis: Calcd. for ($C_{20}H_{16}FN_3OS$) C : 65.74, H:4.41, N:11.50, S:8.77 . Observed: C: 64.83, H:4.23, N:10.92,S:7.97. ^{1}HNMR (500MHz, $CDCl_3$, δ=ppm); 7.29-7.02 (m, 8H, Ar-H +1H,CH=C-*alkene*); 4.53 (1H, q, CH-*aliph.);* 2.03-1.24 (6H,m, 2xCH_3).

Molecular docking study

All the molecular docking studies were carried out using MOE, 2014 software. With a force field tool, energy minimization was executed with (RMSD) gradient of 0.05kcal/mol (Brooks *et al*., 2009), errors were fixed and the partial charges were computed. The protein data bank file (PDB:

(2OH4) (El-Feky *et al*., 2015) was selected for the pocket of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). The enzyme was prepared for docking studies through the following steps (i) water molecules were removed (ii) adding of hydrogen atoms to the enzyme; (iii) the preparation of the reference ligand sorafenib (iv) the removal of the co-crystallized ligands and docking of the new targeted structures (Coutsias *et al*., 2004).

Biological screening

The study of MTT and HCS was carried out using the procedure outlined earlier in the literature (Sebeka *et al*., 2017; El-Far *et al*.,2009).

MTT assay

The MTT assay would be used to estimate the cytotoxicity of the synthesized compounds **(3-7)** and compounds **(9-16)** against MCF-7 and WRL-68 normal cell lines that use sorafenib as a reference drug.

Dimethyl-sulfoxide (DMSO.), was added to each of the synthesized compounds, to prepare a stock solution and serial dilutions (6.25-400µg/mL). 100µL of each of the samples and sorafenib (positive control), were added to MCF-7, and WRL-68 cell lines, and the cell culture was kept in the CO_2 incubator at (37°C) for 24h. Addition of 20mL of MTT stock solution (6µg./ mL) to each well and plates followed, then incubation for 1-4h ,when the media was removed, DMSO was already introduced per each well to dissolve the crystals of the formazan .The absorbance was validated at 575nm, through the use of a Hidex-Chameleon-microplate reader (Lab Logic Systems- Ltd, Sheffield, United Kingdom). The small portion of the absorbance of the cells treated with the synthesized compounds compared to the absorbance of untreated cells, (negative control) was calculated as a percentage of cell viability. The IC_{50} values were calculated for each target compound (Abraham *et al.,* 2008; Hmood *et al*. 2020).

High-Content Screening (HCS)

Three kits of Cellomics multi parameter cytotoxicity (Thermo Scientific, Japan) were also used to detect viable cell counts, nuclear, intensity, cell membrane permeability, mitochondrial membrane potential, and cytochrome C levels in the MCF-7 cell lines after treatment with compound (**15**). Briefly. 24h after compound's **15** treatment, mitochondrial membrane potential (MMP) dye, and the cell permeability dye were already added to the cells and allowed to stand for 30min at 37°C. Cells were already fixed, permeabilized, and blocked with 1X blocking buffer prior probing with primary cytochrome C antibody and secondary DyLight TM 649 conjugated goat anti-mouse IgG for one h each. The plates were assessed by using the ArrayScan high content screening (HCS) system. A detailed study was carried out earlier in the literature (Looi *et al*., 2013).

Caspases 7 and 9 Activity Assay

The caspase activity assay was performed with the use of of Caspase Glo7 and 9 kit (Promega-Madison-WI (Hamed *et al*., 2014). Briefly, MCF-7 cells were grown into 96-well plates, with various concentrations of compound (1**5**) added for 24h. 100μL of Glo 9 reagent was added and allowed to stand for 30min at 25°C, the effect of the caspase in the treated cells was determined by the extent of amino-luciferin-labeled tetra peptide cleavage, and luciferase enzyme substrates release, using a Tecan-Infinite 200 Pro micro plate readers. Furthermore, time-dependent mode experiments were also performed to validate the expression degree of caspases and hence, MCF-7 cells had been treated with compound (1**5**) with different concentrations. Then the assessment of the degree of expression of caspases 7 and 9 at different times was carried out (Paydar *et al*., 2014).

Statistical analysis

One way analysis of variances (ANOVAs) was used to compute whether the variances were statistically significant; data were calculated as the mean ± standard deviation, and statistically relevant results were analyzed using Graph Pad PrismV6 (Graph Pad program, Inc.).

RESULTS AND DISCUSSION

Docking study

Molecular docking processes

Molecular docking studies have been shown that the target compounds may be considered as potential inhibitors of the Vascular Endothelial Growth Factor (VEGFR-2) receptor. Vascular endothelial growth factor (VEGF) is over expressed in MCF-7 cell, and it is one of the most vital angiogenic factors associated with the development of the cancers.

All experimental models were produced using MOE 2014.0901 software. Throughout every experiment, cells that express VEGFR-2 were obtained from the Protein Data Bank (PDB ID 2OH4) (El-Feky *et al*., 2015).

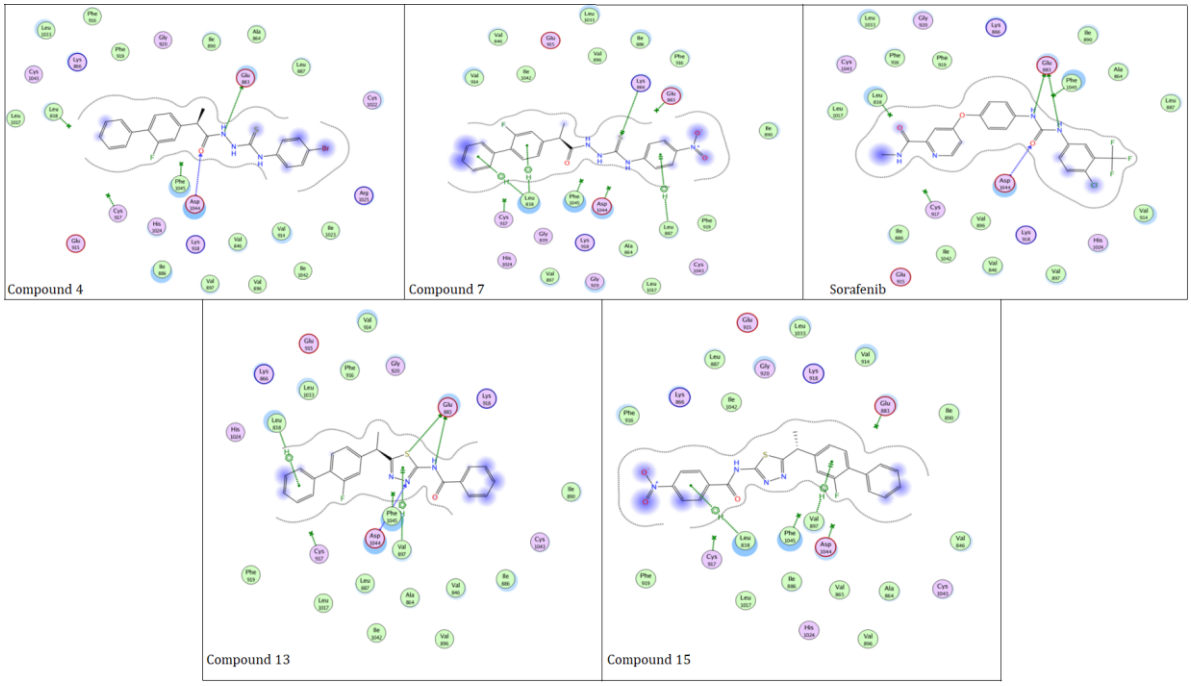


Figure 3. 2D Crystal ligand of some synthesized compounds (4,7,13 and 15), and sorafenib as potent inhibitor against VEGFR-2 pocket

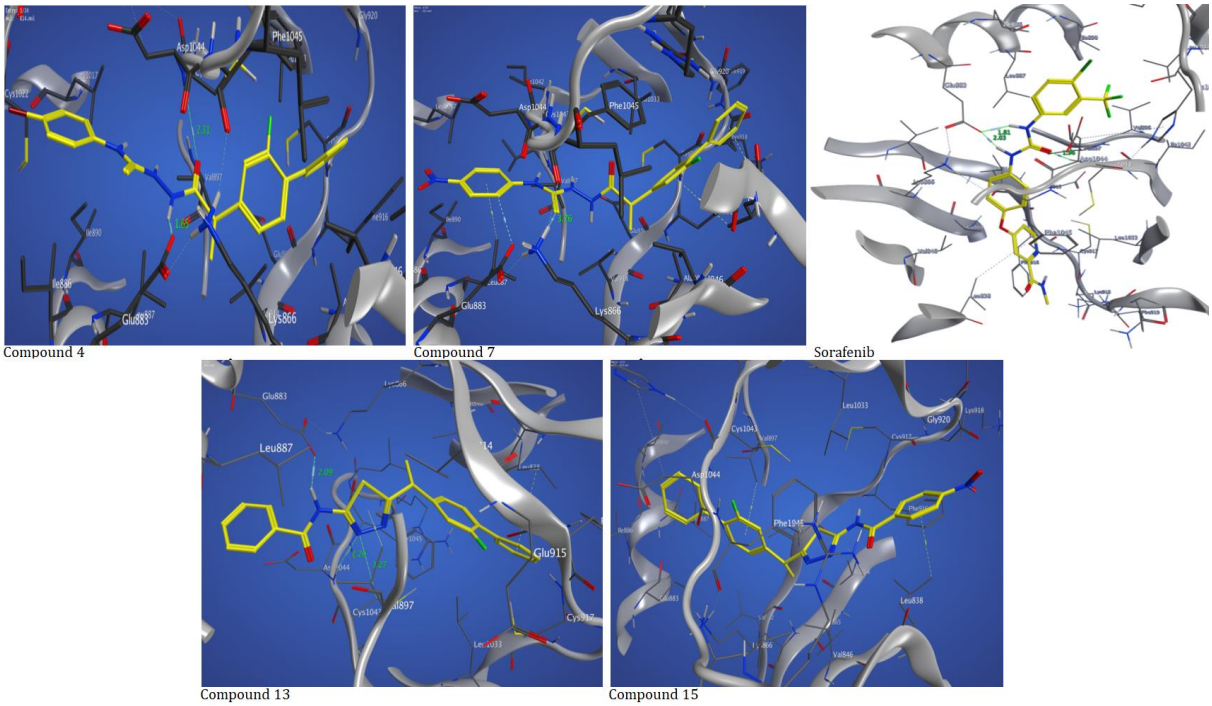


Figure 4. 3D binding mode of some synthesized compounds (4,7,13 and15) and sorafinib (Yellow) docked in crystal protein of VEGFR-2 (PDB: 2OH4) and the corresponding interaction diagram, hydrogen bonds with green color and *pi*-interaction with gray color.

Table I. Show (ΔG) kcal/mol of the tested compounds against (VEGFR-2) target site PDB ID: 2OH4.

Comp. No.	Bonds		Interaction with key amino acids	Score (ΔG) Kcal /mol	RMSD value	E-place	Bonds length range/Å
	H.B	*pi*					
3	2	1	Glu883-Asp1044	-4.22	1.37	-96.61	1.90-1.98
4	2	0	Glu883-Asp1044	-6.77	3.58	-80.96	2.31-165
5	2	1	Glu883-Asp1044-Cys917	-5.02	1.03	-70.33	1.62
6	2	0	Glu883-Asp1044	-3.24	1.56	-60.52	2.95-2.85
7	1	3	Lys866-Leu887-Leu838	-6.12	1.09	-99.70	3.76
9	1	2	Leu838-leu887-Glu883	-3.48	1.91	-94.86	1.73
10	0	2	Val 897-Leu838	-4.65	1.75	-88.38	-
11	0	2	Val 897-Leu838	-5.09	1.70	-85.69	-
12	0	2	Phe1045-leu838	-3.89	0.88	-114.8	-
13	3	2	Glu883-Asp1044-Val897	-6.68	2.05	-89.9	2.09
14	1	0	Asp1044	-6.59	1.98	-97.69	1.71
15	0	2	Val897-Leu838	-6.43	2.16	-110.39	-
16	1	1	Cys917-Val 897	-1.17	0.79	-83.8	2.13
Sorafenib	3	0	Glu883-Asp1044	-9.71	1.22	-110.87	1.81-2.03

Table II. Predicted ADMET analysis for the target compounds (3-16).

Comp. No.	BBB level	HIA	CYTP450 3A4	CYP2D6	CYP2C9	PPB	Solubility level
3	4.120	94.59	0	0	0	98.20	1
4	5.720	95.24	0	0	0	91.60	1
5	5.550	93.03	0	0	0	95.50	1
6	3.220	94.87	0	0	Inhibitor	96.40	2
7	0.510	93.41	0	0	inhibitor	97.70	2
9	0.708	96.31	0	0	0	92.77	2
10	1.830	97.30	0	0	0	91.10	2
11	1.670	79.02	0	0	0	91.51	3
12	0.036	69.81	0	0	0	92.5	2
13	0.048	96.61	0	0	0	93.9	2
14	0.043	96.6	0	0	0	93.33	2
15	0.580	98.16	0	0	0	91.07	1
16	0.047	97.70	0	0	0	92.96	2

Solubility level: (4) high sol. , (3)and (2) intermediate sol. , (1) less sol. , (0) poor sol; BBB: Blood-Brain Barrier; HIA: Human Intestinal Absorption; CYP2D6: Cytochrome P2D6, 0= non-inhibitor; PPB: Plasma Protein Binding.

The results showed that all ligands investigated had a quite comparable position and orientation within the defined binding site of VEGFR-2, which explains a vast area bounded by a membrane-binding domain.

The outcome of the free energy of binding (ΔG) stated that some of these compounds might have high binding affinity toward the receptor, and the computed values reflected the general pattern, (Table I). The key binding sites of (VEGFR-2) was reported in the literatures(El-Adl *et al*., 2020), consisting of amino acids Tyr644, Ile619, Leu746, Thr692, Lys646, Glu706, Lys702, and Glu663.

At a distance of 1.65°A.The binding mode of compound **4**, revealed an energy binding of –6.77 kcal/mol. The nitrogen group at hydrazide moiety formed a hydrogen bond with Glu883. Also, the (C=O) group formed a hydrogen bond with Asp1044 at a distance of 2.31°A.

Compound **7** revealed binding energy of –6.12 kcal/mol. The sulfur group at thiourea moiety formed a H-bond with Lys866 at a distance of 3.76°A. While the hydrophobic phenyl rings formed three *Vander Waals* interactions, one with Leu887, and two bindings with Leu838.

Table III. Carcinogenicity prediction of the synthesized compounds (3-16).

Comp. No.	Ames test	Carcinogen on mouse	Carcinogen on rat	HERG inhibitor	Carcinogenicity	TA100-NA
3	Mutagen	Positive	Positive	High risk	1	-
4	Mutagen	Positive	Negative	-	0	-
5	Mutagen	Positive	Negative	High risk	0	-
6	Mutagen	Positive	Negative	Low risk	0	-
7	Non Mutagen	Negative	Negative	-	0	-
9	Mutagen	Positive	Negative	high risk	1	-
10	Mutagen	Positive	Positive	Med. Risk	1	-
11	Mutagen	Positive	Negative	Med. Risk	0	-
12	Mutagen	Negative	Negative	high Risk	1	-
13	Mutagen	Positive	Negative	Med. Risk	0	-
14	Mutagen	Negative	Positive	Med. Risk	0	-
15	Mutagen	Negative	Negative	Med. Risk	0	-
16	Mutagen	Positive	Positive	Med. Risk	0	-

carcinogen = 1; may be non-carcinogen = 0

Table IV. C log p, molecular volume (MV), topological polar surface area (TPSA), and percent of absorption of the synthesized compounds

Comp. No	Clogp	MV	*n*-Violation	TPSA	% *(Abs)
3	4.92	349.23	0	53.14	91
4	5.73	367.12	1	53.15	91
5	5.60	362.77	1	53.15	91
6	4.98	377.78	0	62.39	87.5
7	4.88	372.57	0	98.98	75
9	5.32	371.60	1	99.89	74.5
10	6.13	513.46	2	49.84	92
11	6.00	385.19	1	49.15	92
12	5.28	344.49	1	95.66	76
13	5.00	350.57	1	54.88	90
14	5.16	433.51	1	64.12	87
15	5.06	377.70	1	100.71	74.25
16	4.23	312.48	0	47.7	92.5

%*(Abs.) calculated according to a method described by (*Zhao,2002 et al.,*).

Compound **13** presented an energy binding of –6.68 kcal/mol. The thiadiazole ring formed two H-bonds with Glu883 and Asp1044 at a distance of 2.25 Å. Also, the secondary amido (NH) group formed a H-bond with Glu883 at a distance of 2.09^{0}A. While the phenyl and thiadiazole rings formed two hydrophobic interactions with Leu838, and Val 897, respectively. The mode of binding of compound **15** exhibited an energy binding of 6.43kcal/mol. The *p*-nitro phenyl ring, and *m*-flouro phenyl ring formed two hydrophobic interactions with Leu838 and Val897, respectively. Finally, It is important to mention the binding mode of sorafenib, as a reference, which exhibited an energy binding of –9.71 kcal/mol. The amino group at urea moiety formed two H-bonds with Glu883 at a distance of 1.81 –2.03 Å. Also, the (C=O) formed a H-bond with Asp1044 at a distance of 1.9 Å. All the above bindings mode 2D and 3D simulation attachment against (VEGFR-2) critical amino acids, length of hydrogen bonds/Å, are presented in (Table I and Figures 3 and 4).

In silico ADMET and carcinogenicity analysis

The pharmacokinetic profile, i.e. absorption, distribution, metabolism, and excretion was anticipated by ADMET descriptor module of the small molecules protocol of *pre* ADMET online software, http://preadmet.bmdrc.kr/adme/. (Table II).

The synthesized derivatives exhibited higher penetration to blood brain barrier (BBB). In addition, all of them possessed good human intestinal absorption (HIA) from the intestine following oral absorption, the HIA value ranged from 69.81 -98.16 (Eissa *et al*., 2018, Al-Saad *et al*., 2020). It was also noted that their aqueous solubility logarithmic level ranged from (1-3) in most of the compounds, reflecting low to moderate aqueous solubility. The plasma protein binding model showed good binding ability of the synthesized compounds to plasma proteins. All of the marked compounds were found to be non-inhibitors of CYTP450 3A4, CYP2D6 and CYP2C9, except for compounds **6** and **7** which were reported to be inhibitors of enzyme CYP2C9 (Table II).

Finally, (Table III) indicated that the series of the synthesized compounds are non–carcinogenic, except for compounds **3,9,10,** and **12(**carcinogenicity score=1**)** .While, the rest of the compounds are assumed to be safe.

Physico-chemical properties of the synthesized compounds (3-16)

The values of Clogp, the topological polar surface area (TPSA) and molecular volume (MV) were calculated using Chem-informatics on the Web (http:/www.molinspiration.com), (Table IV). The Clog p value gives an indication of the lipophilicity of the chemical compound. The increasing trend in this value leads to a rise in the lipophilic character of the compound tested (Rudraraju *et al*., 2014). It is worth mentioning that the Clog p values for the tested compounds ranged from 4.23 to 6.13. The tested compounds **3, 6, 7**, and **16** are in clear violation of Lipiniski's rule of five. Further, the topological polar surface area (TPSA) of the new derivatives ranged from 47.70 to 100.71, with a reasonable oral bioavailability (Veber *et al*., 2002), and acceptable molecular volume (MV) values. The percentage of absorption (percent Abs.) was determined, according to the method outlined by *Zhao et al*. (Zhao *et al*., 2002) (Table IV).

Pre-metabolism analysis

The finding of the *pre*-metabolism investigation on compound (**13**) was taken as a prototype for the predicted metabolism analysis, revealing that CYTP450 3A4 liver microsomal enzyme had a small influence on the compound tested as CYP2C9 liver microsomal enzyme, with the most metabolic reaction taking place to be benzene hydroxylation, whose product would be relatively easy to execrate it from the human body, (Table V).

Biological study

MTT assay

The synthesized compounds (**3-7**)and (**9-16**) were assessed for cytotoxicity against MCF-7 (human, breast tumor), and one normal cell line WRL-68 (human hepatic cell line), by 3 -(4,5-dimethyl thiazolyl-2)- 2,5- biphenyl tetrazolium bromide, (MTT) assay. Sorafenib was used as a reference drug. The results were recorded as (IC_{50}) (Figure 5). In order to explore the mechanism of action for compound **(15)** inhibition on proliferation of MCF-7 cells, some conclusive factors, related to apoptosis were assessed by an *in vitro* Cytotoxic Multi-Parameter Assay against MCF-7 (human,breast cancer) undertaken in High Content Screening (Array Scan XTI, Thermo Scientific). Such factors include the effect of compound **(15)** on viable cell count, nuclear intensity, cell membrane permeability, mitochondrial membrane potential (MMP), and cytochrome C release, in addition to the five healthy cellular parameters, the effect of compound **(15**) was tested on caspase 9 activity, compared to a positive control (sorafenib).

The analysis revealed the cytotoxic effect of each of the compounds tested at concentrations [12.5 to 400µg /mL] on the viability of MCF-7 and WRL68 cells, after 24h of treatment, where all of these compounds **(3-16)** resulted in a substantial reduction in the survival rate of WRL68 and MCF-7 cells in concentration-dependence way, ($P<0.0001$) (Figure 6 and Table VII). Overall, the results showed that the synthesized compounds substantially improve the cytotoxicity against the breast cancer cell (MCF-7) with acceptable selectivity index, (SI) (Şenkardeş *et al*., 2020). Compounds **(3,6 and7**) were more toxic toward normal WRL-68 cells, their selectivity indices (SI) were 0.59, 0.69 and 0.87,respectively, compared to sorafenib with (SI) equal to 2.5. Compounds (**11,13,15 and 16)** were more selective toward MCF-7, and their (SI) were 2.55,1.99,2.10 and 3.0**,** respectively ,and possessed the highest potency (lowest IC_{50} values), compared to their effect on normal cell (WRL-68), with the lowest potency (highest IC_{50} values); therefore, these compounds are more selective for the type of breast cancer cell (MCF-7) (Table VI).

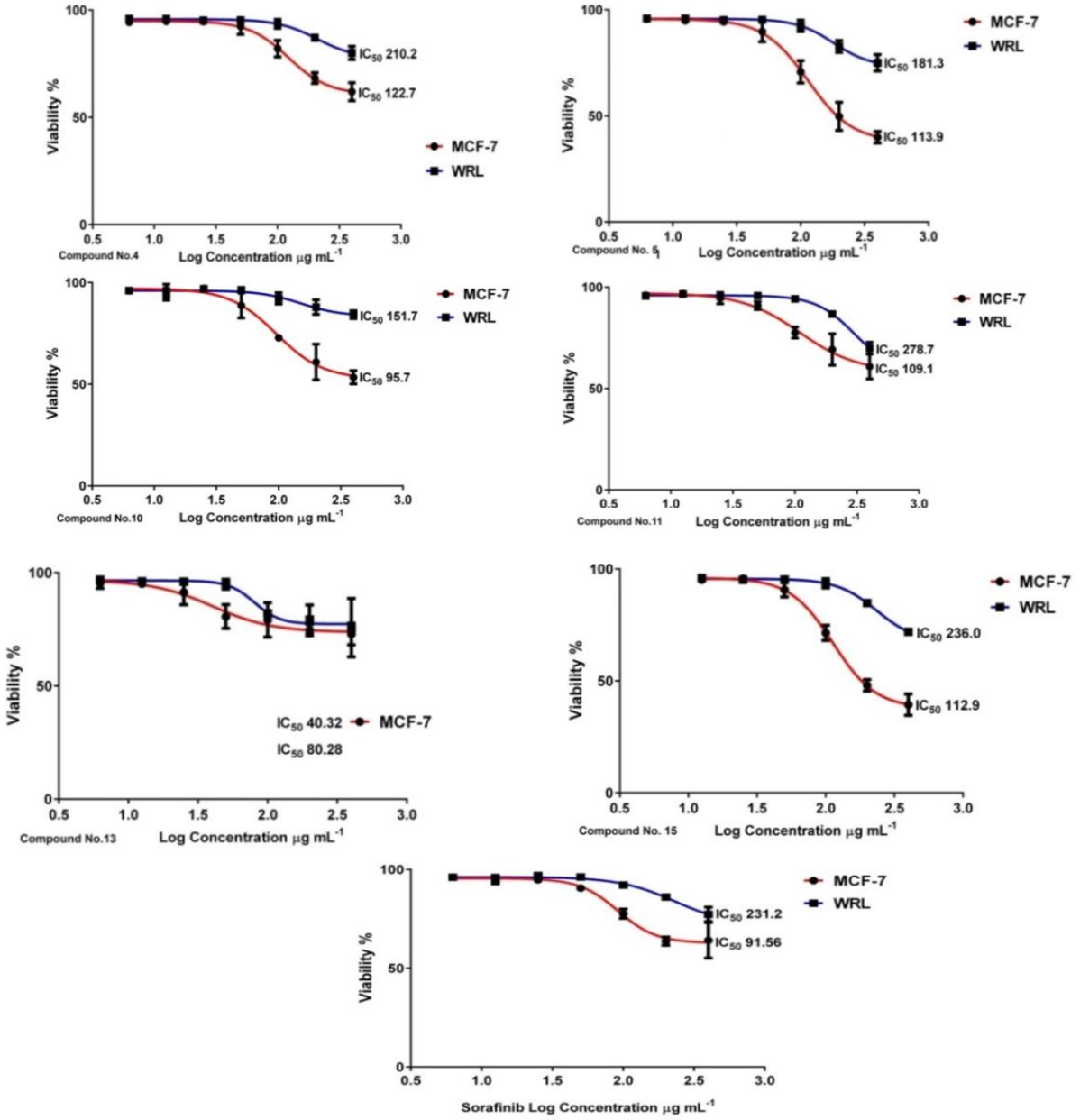


Figure 5. Dose-dependent cytotoxic effect of some synthesized compounds (4,5,10,11,13 and15), and sorafenib on MCF-7 and WRL-68 cells after 24h incubation at 37°C.

Multiple cytotoxic effects of compound (15) on MCF-7 cells *via* array scan high content screening

The cytotoxic effect of compound **(15)** on MCF-7 cell line over 24h was investigated by Array Scan High Content Screening (HCS). Six serial concentrations of compound **(15)** ranged from (200μg/mL to 6.25μg/mL) were prepared and then used to observe the impact on MCF-7 cell viability, nuclear -intensity, membrane -permeability, mitochondrial- membrane potential (MMP), and cytochrome C. The change in such five parameters was shown as mean±SD (Figure 6 and Table VII).

The most major changes in the five parameters were seen at concentrations of 200μg/mL of compound **(15),** compared to sorafenib, (200μg /mL, $p < 0.0001$).

Table V. Show effect of liver microsomal enzymes on compound (13)

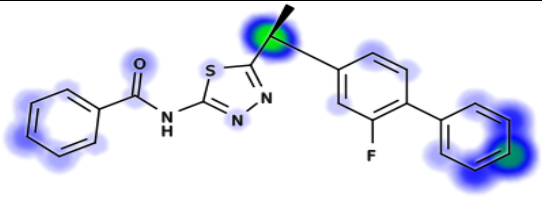
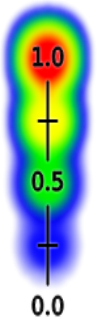
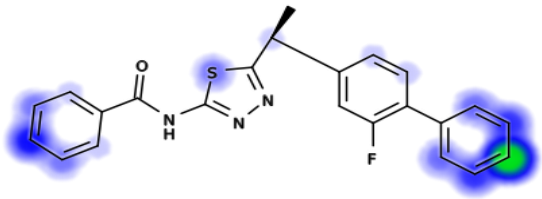
Enzyme	CYT. P450 enzymes effect on compound (13)	Reference scale bar
3A4		
2C9		

Table VI. The IC_{50} values determined by MTT assay against MCF-7 and normal WRL-68 cells.

Comp. No.	IC_{50} µg/mL		*Selectivity Index (SI)
	WRL-68	MCF-7	
3	136.5	231.5	0.59
4	210.2	122.7	1.71
5	181.3	113.9	1.59
6	136.5	198.6	0.69
7	175.5	200.8	0.87
9	190.0	152.6	1.25
10	151.7	95.7	1.59
11	278.7	109.1	2.55
12	69.1	67.53	1.02
13	80.28	40.32	1.99
14	185.1	130.8	1.42
15	236	112.29	2.10
16	392	129.9	3.00
Sorafenib	231.2	91.56	2.50

*SI: IC_{50} on normal WRL-68 /IC_{50} on cancer MCF-7 cell (Şenkardeş *et al.*,2020)

Viable cell count

Compound (**15**) caused a significant reduction in viable cell count only at (200 and 100µg/mL), (p<0.0001 and p=0.0003, respectively), while at concentrations ranging from (6.25-50 µg/mL),the MCF-7 cell lines were not considerably decreased in count, (P≥0.7003)**.** The reduction trend in cell viability was 27.25% at [200µg/mL], 21.6% at [100µg/mL], 9.7% at [50µg/mL], 2.9% at [25µg/mL], 3.3% at [12.5µg/mL], and 1.3 % at [6.25µg/mL], compared to (sorafenib), 59% at [200µg/mL], and to the negative control (untreated cell) (Figure 6A and Table VII). Comparative study was previously achieved, following the treatment of sorafenib analogs to MCF7 cells (Wu *et al.*, 2015).

Nuclear intensity

The influence of compound (**15**) on nuclear intensity (MCF-7), (Figure 6B and Table VII), where this compound has been shown to raise the nuclear intensity of MCF-7, at concentrations of 200µg/mL, 100µg/mL, 50µg/mL, 25µg/mL, 12.5µg/mL, and 6.25µg/mL. The change in the mean nuclear intensity was 833.0±61.54; 676.7±48.99; 509.7±19.04; 464.0±11.14; 504.0±16.7 and 421.3±8.145, respectively, compared to sorafenib, that mostly produced 956.7±39.53 increase in the nuclear intensity, and against a negative control, (untreated cells) of 427.0±44.93.

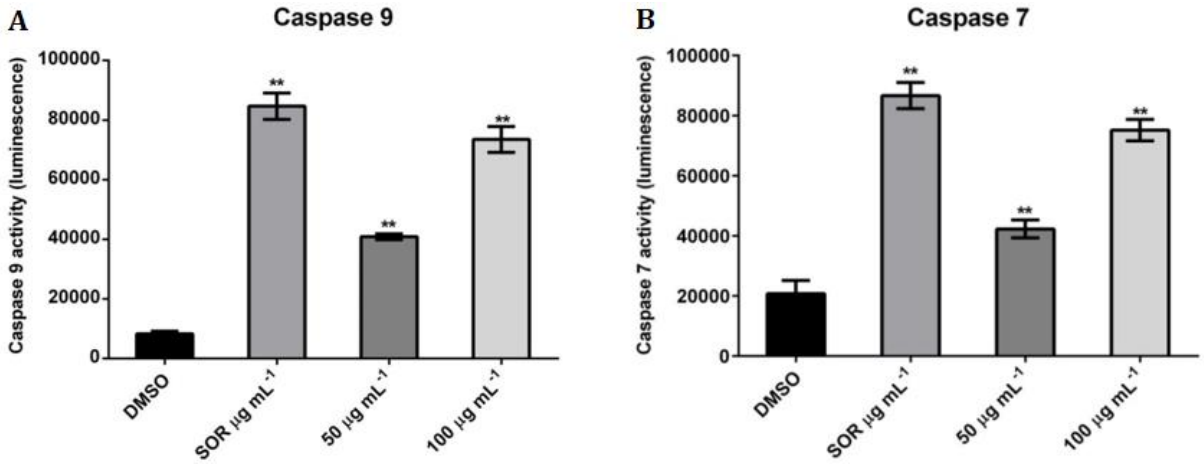


Figure 7 A. The Caspase-9 luminescence of MCF-7 treated with compound (15); B: Caspase-7 activity in MCF-7 cells treated with compound (15) after incubation for 24 h at 37°C.

Table VII. Multiple cytotoxic effects of compound (15) on five cell parameters of MCF-7 cell line via Array Scan high content screening (HCS).

Cytochrome C release	Mitochondrial membrane potential (MMP)	Cell permeability	Nuclear intensity	MCF-7 Cell inhibition %	Concentration (µg/mL)
215.0±22.91	428.3±41.20	79.33±14.05	427.0±44.93	0	Untreated cell
369.7±7.506 **	312.3±8.083 **	90.0±7.81	833.0±61.54 **	27.25 **	200
300.3±17.04 **	348.3±38.66 **	79.33±2. 082	676.7±48.99	21.60 **	100
252.7±23.18	419±29.02	61.00±12.53	509.7±19.04	9.70	50
208.7±21.13	437±12.49	72.00±8.544	504.0±16.70	2.90	25
221.7±3.786	436±9.165	80.33±1.155	464.0±11.14	3.30	12.5
213.3±26.58	438±16.7	78.00±4.359	421.3±8.145	1.30	6.25
654.7±50.65 **	106±12.77 **	203.7±30.66 **	956.7±39.53**	59.00 **	Sorafenib

Table VIII. The caspases-9 and-7 activities in MCF-7 cells treated with compound (15) after incubation for 24h at 37°C .

Concentration µg/mL	Caspase 9 (mean± SD), MCF-7 cell line	Caspase 7 (mean ± SD),MCF-7 cell line
50	40855+949.8**	42288+2997***
100	73502+4344**	73169+3590***
Sorafenib	84652+4344**	86652+4356***
DMSO	8317+885	790+4391

**refers to significant result at $p < 0.0001$; ***refers to significant result at $p \leq 0.0004$

The effect of compound (**15**) on the MCF-7 cell line with a concentration of more than 50µg/mL) was concentration-dependent, while at lower concentrations, (50, 25, 12.5 and 6.25µg/mL), there was no real effect on nuclear intensity, ($p \geq 0.0657$). In the previous study, comparative data were obtained following treatment of MCF7 cells with the anastrozole (Hassan *et al*., 2018).

Cell membrane permeability

The compound (**15**) effect on MCF-7 cells was shown with non-significant effects at all concentrations, therefore, compound (**15**) did not posses any effect on the cell membrane integrity, compared to sorafenib, as a positive control, that

resulted in a remarkable concentration effect at (200µg/mL), (203.7±30.66), (p<0.0001) (Figure 6C and Table VII).

Mitochondrial membrane potential (MMP)

The effects of compound (**15**), on the changes in the permeability of the mitochondrial membrane were evaluated in this experiment. The intensity of MMP was decreased with rising compound's (**15)** concentration in a concentration-dependent manner. At 200µg/mL and 100µg/mL, there was a significant decrease in the mean of the intensity of MMP activity (312.3±8.083, 348.3±38.66) respectively, p<0.0001), compared to sorafenib at (200µg/mL, p<0.0001), there was a decrease in the mean of the intensity of MMP to 106±12.77. Low concentrations of compound (**15**), less than <100µg/mL, resulted in a non-significant effect (p=0.9971) (Figure 6D and Table VII).

Cytochrome C release

The results of cytochrome release for compound (**15**)in MCF-7 cell line were a concentration-dependent. There was a marked raise in the cytochrome C release, (p<0.0001), with an increase in the concentrations, compared to a negative control, (215.0±22.91); where, the concentrations of compound (**15**) at 200µg/mL, 100µg/mL, caused a detectable changes in mean nuclear intensity as follows: (369.7±7.506, 300.3±17.04), respectively, (p<0.0001). While at low concentrations below <100µg/mL, there was a non-significant effect (p=0.3433).

Sorafenib also caused changes in mean nuclear intensity, 654.7±50.65 (p<0.0001) (Figure 6 E and Table VII).

Effects of compound (15) on MCF-7 cell caspase 9

The outcomes of caspase-9 activity of MCF-7 treated with compound (**15)** (Figure 7); and there was a substantial increase in caspase 9 activity after 24h, which was (40855±949.8, 73502±4344), (p<0.0001) in MCF-7 cells treated with 50µg/mL, and 100µg/mL of compound **(15),** respectively, compared to sorafenib which was set to increase caspase 9 activity to 84652±4344, (p<0.0001), and in a non-treated cell (DMSO) to 8317±885, (Figure 7A and Table VIII).

Evaluation of caspase- 7 activity in MCF-7 cell treated with compound (15)

The results of caspase-7 activity showed that there is a significant increase in caspase 7 activity after 24h, where the activity was, (42288±2997, and 73169±3590), (p<0.0001, and p<0.0004) at MCF-7 cells treated with 50µg/mL, and 100µg/mL of compound (15), respectively, compared to sorafenib that produced raise in caspase 7 activity to 86652±4356,(p<0.0001) ,and in a non-treated cell (DMSO), to 20790±4391 (Figure 7B and Table VIII).

These results showed that MCF-7 cells treated with compound **(15)** undergo apoptosis by disrupting the mitochondrial membrane and release of cytochrome C (Choo *et al.,* 2017).Since these two occurrences are closely correlated to caspase activation. Caspase 9 is a downstream initiate for the activation of another caspases (e.g. 3 and/or 7) leading to apoptosis (Loong *et al*., 2014).

CONCLUSIONS

New compounds (**3-16**) were synthesized and approved by spectroscopic analysis including FTIR, ^{1}HNMR and CHNS. The defined compounds exhibited anticancer activity against the MCF-7 cell line with a maximum effect expressed by compounds **11** and **15** at their (IC_{50}) , 112.9 and 109.1µg/mL, respectively, and with low effect on (WRL-68) cells. Treated MCF-7 tumor cells at different concentrations with such a compound (**15**) observed statistically measurable reduction in valid cell count, loss of mitochondrial membrane potential (MMP), and a remarkable increase in total nuclear intensity, mitochondrial release of cytochrome C, and increase in caspase 7 and 9 activity. Experiments on molecular docking have shown that target compounds may be considered highly plausible inhibitors of the Vascular Endothelial Growth Factor (VEGFR-2) receptor.

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