Synthesis, molecular modeling study, preliminary antitumor and antimicrobial evaluation of new benzimidazole derivatives

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Abstract: New benzimidazole derivatives have been synthesized and tested for their antineoplastic activity employing three cancer panels; HepG2, MCF-7 and Vero B by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. In addition, antimicrobial activity was evaluated using disk diffusion assay and two-fold serial broth dilution method. Only compound **(3)**, 2-(1-phenyl-1*H*-tetrazol-5-ylthio)-1*H*-benzimidazole, showed antitumor activity against MCF-7 with IC_{50} of 30 µg/ml. However, 5-(2-(2-(1-phenyl-1*H*-tetrazol-5-ylthio)-1*H*-benzimidazol-1-yl)ethyl)-1,3,4-oxadiazole-2-thiol **(6)** and 4-phenyl-5-(2-(2-(1-phenyl-1*H*-tetrazol-5-ylthio)-1*H*-benzimidazol-1-yl)ethyl)-4*H*-1,2,4-triazole-3-thiol **(7)** exhibited significant antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*. Moreover, vitamin D3 receptor and dihydrofolate reductase enzyme were chosen as targets for molecular modeling study concerning antitumor and antimicrobial activity, respectively. The detailed synthesis, spectroscopic, molecular modeling and biological data are reported.
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1. Introduction

Cancer is a relatively autonomous uncontrolled growth of cells which proliferate in an irregular manner. It is continuing to be a major health problem in the world (Xiaomei and Herbert 2006). The expected number of people, who would die of cancer by the year 2020, is anticipated to be 10 millions, as mentioned by Schumacher (Schumacher *et al*., 2011). In addition, microbial infections still remain a major health threat in underdeveloped countries. In the course of identifying various chemical substances which may serve as a lead for designing novel antitumor and antimicrobial agents, we are particularly interested in the present work with benzimidazole derivatives. Benzimidazoles were widely explored for development of anticancer agent being an isostere of purine based nucleic acid and an important scaffold in various biologically active materials (Bansal and Silakari 2012). Varied substituents around the benzimidazole nucleus have provided a large number of derivatives with antitumor activity (Mahiuddin *et al*., 2007).

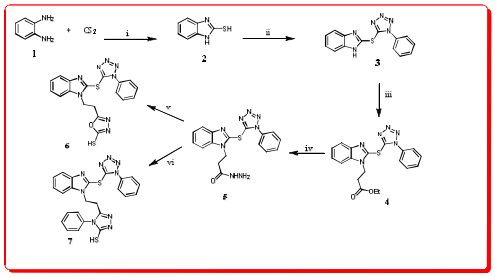
Moreover, attention has increasingly been given to the synthesis of benzimidazole derivatives as a source of new antimicrobial agents as revealed by literature survey (Namrata *et al*., 2012). In addition, certain 1,2,4-triazoles (Nadeem *et al*., 2011), 1,3,4-oxadizoles (Monika and Shivi 2011) and tetrazoles (Somisetti *et al*., 2012) exhibit good antimicrobial activity. Epidemiological studies indicated that vitamin D insufficiency could have an etiological role in various human cancers (Kristen *et al*., 2007). Preclinical research indicated that the active metabolite of vitamin D, known as Calcitriol, might have potential as anticancer agent because its administration has antiproliferative effect, can activate apoptotic pathway and inhibits angiogenesis (Aruna *et al*., 2010). However, clinical studies revealed that dihydrofolate reducatse enzyme is considered as important target for antimicrobial drugs belonging to the class of antimetabolites as the enzyme plays an important role in the *de novo* purine synthesis (Moni and Prem 2012).

In the view of the above mentioned findings, new benzimidazole derivatives with new functionalities at 1 and 2 positions have been designed and synthesized aiming at finding new potent antineoplastic and antimicrobial agents. Moreover, vitamin D3 receptor and dihydrofolate reducatse enzyme were chosen as targets in molecular study for antitumor and antimicrobial activities, respectively. This choice was based on the recorded high fit score of the synthesized compounds against these targets obtained by PharmMapper.

2. Results and discussion

2.1. Chemistry

The target compounds were synthesized as depicted in **Scheme I**.



Scheme I Reaction protocol for the synthesis of **2-7** (i) KOH; (ii) 5-Chloro-N-phenyltetrazole, K_2CO_3, DMF; (iii) $BrCH_2CH_2COOEt$, NaH, THF; (iv) Hydrazine hydrate, absolute ethanol; (v) CS_2, KOH, 95% ethanol; (vi) Phenylisothiocyanate, KOH, 95% ethanol.

The reported key intermediate 2-mercapto benzimidazole **2** was prepared by refluxing *o*-phenylenediamine **1** with carbon disulfide in the presence of potassium hydroxide (Heralagi *et al.*, 2012). This intermediate was then alkylated with 5-chloro-N-phenyltetrazole in the presence of potassium carbonate using dimethylformamide as a solvent to give 2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazole **3**. Heating of **3** with ethylbromopropionate in tetrahydrofuran in the presence of sodium hydride gave ethyl-3-(2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazol-1-yl) propanoate **4**. The IR spectrum of **4** revealed the presence of carbonyl band at 1744 cm^{-1} and the absence of NH-band at 3320 cm^{-1}. Reaction of compound **4** with hydrazine hydrate in absolute ethanol afforded the acid hydrazide derivative **5** in an excellent yield. The oxadiazole **6** and the triazole **7** moieties were constructed by refluxing the acid hydrazide **5** with carbon disulfide and phenylisothiocyanate, respectively in ethanol in the presence of potassium hydroxide as a base. The structures of the compounds formed were confirmed by IR, mass spectroscopy, ^{1}H-NMR and elemental analysis.

2.2. Molecular Modeling

2.2.1. Cytotoxic activity

A primary molecular modeling study of the newly synthesized derivatives was performed by submitting their structures to the free online site PharmMapper (Xiaofeng *et al.*, 2010) to get different binding energy scores with different targets involved in cancer. Chemical structures were firstly converted into mol2 file format, then submitted to the site. Results from the site were received and carefully analyzed to obtain a conclusive idea about the most appropriate targets of the examined compounds.

The newly synthesized compounds **3-7** were found to have promising anticancer activity as revealed by their high fit score with vitamin D3 receptor whose PDB_ID is (1S0Z), since recent observations indicate that vitamin D insufficiency could have an etiological role in various human cancers. The scores that indicate the interaction energies of these compounds to 1S0Z are illustrated in table 1.

Docking calculations were then carried out using the online site DockingServer (Bikadi and Hazai 2009), which perform docking using AutoDock 4.0 (Huey *et al.*, 2007). Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Essential hydrogen atoms, Kollman united atom type charges and solvation parameters were added with the aid of AutoDock tools (Morris and Goodsell 1998). Affinity (grid) maps of 20 Å grid points and 0.375 Å spacing were generated using the Autogrid program. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis and Wets local search method. Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluation. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

Table 1. The Energy fit score of compounds 3-7 to 1S0Z.

Compd. No.	Energy fit score
3	2.609
4	2.102
5	2.203
6	3.458
7	3.849

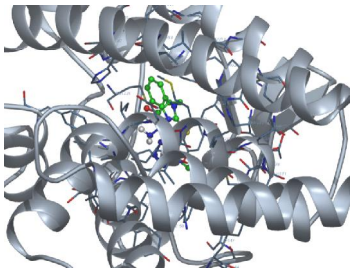
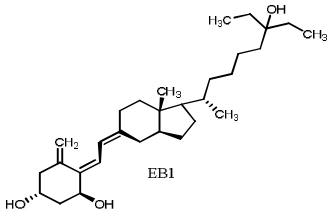


Figure 1. Compound **6** docked into the active site of vitamin D3 receptor

Table 2. Results of Docking of test compounds 3-7 to 1S0Z.

Compd. No.	Est. Free Energy of Binding Kcal/mol	Est. Inhibition Constant, Ki	vdW + Hbond + desolv. Energy Kcal/mol	Electrostatic Energy Kcal/mol	Total Intermolec. Energy Kcal/mol
3	-8.51	580.38 nM	-9.18	-0.04	-9.22
4	-7.88	1.67 uM	-9.42	-0.01	-9.42
5	-8.97	267.09 nM	-10.27	-0.02	-10.29
6	-9.44	120.55 nM	-10.70	+0.05	-10.66
7	-7.89	1.64 uM	-9.24	+0.01	-9.23
Reference ligand EB1 A	-10.68	14.80 nM	-13.57	+0.02	-13.55

Table 2 illustrates the results obtained from the docking calculations for test compounds and the reference drug, EB1 which acts on the same target.



EB1

The docking studies have revealed that compound **6** is the most active among the tested compounds when compared to the reference drug, figure 1.

2.2.2. Antimicrobial activity

The test compounds **3-7** were submitted to the free online site PharmMapper and their results are shown in table 3. The results showed that the highest energy fit scores were for the target, dihydrofolate reductase enzyme whose PDB_ID is (1DRF). It is an important target for antimicrobial drugs belonging to the class of antimetabolites as the enzyme plays important role in the *de novo* purine synthesis .Thus, this enzyme represents an attractive target for the development of new broad-spectrum antibacterial agents.

Using the previously mentioned DockingServer, the test compounds were submitted, prepared and docked on the prepared protein (1DRF). The results obtained were compared to the antibacterial agent, trimethoprim, which acts on the same target protein, and are shown in table 4.

The docking studies have revealed that compound **6** is the most active among the test compounds when compared to the reference drug, figure 2.

Table 3. The Energy fit score of compounds 3-7 to 1DRF.

Compd. No.	Energy fit score
3	2.524
4	2.324
5	2.566
6	3.567
7	3.324

Table 4. Results of Docking of test compounds 3-7 to 1DRF.

Compd. No.	Est. Free Energy of Binding Kcal/mol	Est. Inhibition Constant, Ki	vdW + Hbond + desolv. Energy Kcal/mol	Electrostatic Energy Kcal/mol	Total Intermolec. Energy Kcal/mol
3	-8.46	633.19 nM	-8.86	+0.01	-8.86
4	-8.31	808.22 nM	-10.06	-0.02	-10.08
5	-9.15	196.78 nM	-10.43	-0.00	-10.43
6	-11.19	6.27 nM	-11.84	-0.23	-12.08
7	-9.84	61.18 nM	-11.79	-0.11	-11.90
Trimethoprim	-4.11	977.26 uM	-3.991	-1.97	-5.96

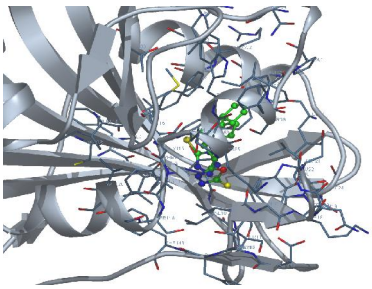


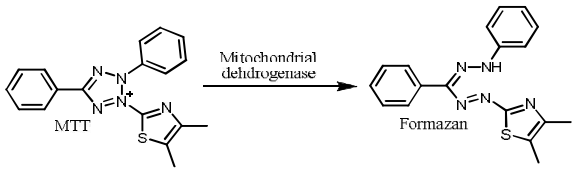
Figure 2. Compound **6** docked into the active site of dihydrofolate reductase enzyme

2.3. Biological activities

2.3.1. Cytotoxic activity

The newly synthesized compounds were screened for their *in vitro* cytotoxic and growth inhibitory activities against three tumor cell lines; HepG2 (human hepatocellular carcinoma cell line), MCF-7 (human breast adenocarcinoma cell line) and Vero B (kidney of normal adult African green monkey) using DMSO as a negative control. The *in vitro* MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl -2*H*-tetrazolium bromide) assay of cytotoxic activity

was employed and the results are shown in Table 5. MTT assay is used to measure the viability of the cells and it is a colorimetric assay that utilizes a yellow tetrazolium dye. This dye is reduced to purple formazan in the mitochondria of living cells in the presence of mitochondrial dehydrogenase enzyme.



Thus, this conversion can be directly related to the number of viable cells and inversely related to the cytotoxicity of the compounds tested. When the amount of the purple formazan produced by cells treated with the synthesized compounds is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agents in causing death can be deduced. The absorbance of the deeply colored formazan solution can be quantified by using a spectrophotometer.

The cytotoxic activities of the tested compounds were expressed as IC_{50} which is the concentration required for 50% inhibition of cell viability. It is evident that the tested compounds showed no significant antitumor activity, since only compound **3**, with free benzimidazole NH-group, showed activity against MCF-7 with IC_{50} 30µg/ml. In an attempt to increase the activity, substitution of NH group was achieved but unfortunately, this resulted in complete loss of activity as observed with compounds **4**, **5**, **6** and **7**.

Table 5. *In vitro* cytotoxic activity of compounds 3-7

Comp. No.	IC_{50} (µg/ml)[a]		
	HepG2[b]	MCF-7[c]	Vero B[d]
3	Ve[e]	30	Ve[e]
4	Ve[e]	Ve[e]	Ve[e]
5	Ve[e]	Ve[e]	Ve[e]
6	Ve[e]	Ve[e]	Ve[e]
7	Ve[e]	Ve[e]	Ve[e]

[a] IC_{50}: Compound concentration required to inhibit tumor cell proliferation by 50%.
[b] Human Hepatocellular carcinoma cell line.
[c] Human breast adenocarcinoma cell line.
[d] Kidney of normal adult African green monkey.
[e] No toxicity

2.3.2. Antimicrobial activity

The newly synthesized compounds were also subjected to preliminary screening of their *in vitro* antimicrobial activity against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using disc diffusion assay. Gentamycin was used as a reference standard. The test Compounds exhibited good activity and then subjected to a quantitative assay in order to determine their minimum inhibitory concentrations (MICs) using the two-fold serial broth dilution assay. It was observed that compounds **6** and **7** have the maximum activity against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* among the test compounds comparable to that of Gentamycin. However, none of the test compounds showed activity against *Pseudomonas aeruginosa.* The structure activity relationship study among the newly synthesized compounds revealed that incorporation of 1,3,4-oxadiazole or 1,2,4-triazole rings into the benzimidazole moiety at 1-position via ethylene bridge devoloped significantly potent antimicrobial analogues (**6** and **7**), when compared to Gentamycin as a reference drug (table 6).

Table 6. *In vitro* antimicrobial activity of compounds **3-7**

Comp. No.	MIC (µg/ml)[a]			
	Gram positive bacteria		Gram negative bacteria	
	A[b]	B[c]	C[d]	D[e]
3	85	66	75	-[f]
4	88	68	89	-[f]
5	72	-[f]	71	-[f]
6	63	53	65	-[f]
7	67	59	68	-[f]
Gentamycin	58	47	63	39

[a] MIC: The lowest concentration of compound needed for prevention of visible growth of micro-organism.
[b] *Bacillus subtilis.*
[c] *Staphylococcus aureus.*
[d] *Escherichia coli.*
[e] *Pseudomonas aeruginosa*
[f] Not tested for MIC, since gave no inhibition zone in disc diffusion assay.

3. Conclusion

From the results of the present study, some benzimidazole derivatives were synthesized and tested for their antitumor activity using MTT assay and antimicrobial activity using disk diffusion assay and two-fold serial broth dilution assay. The tested compounds showed weak anticancer activity. On the other hand, some of the investigated compounds showed an interesting antimicrobial activity.

4. Experimental

4.1. Chemistry

Melting points were determined using Fisher–Johns melting point apparatus and are uncorrected. IR (KBr-discs) spectra were measured by Nicolet MX-1

FT-IR spectrometer. TLC was performed on silica gel G60F254 (E-Merck, Germany). The ^{1}H NMR spectra were recorded in DMSO-d6 using TMS as an internal standard, on a JEOL Eclipse-400 NMR spectrometer. Mass spectra were measured on JEOL JMS-600H spectrometer. Elemental analysis was carried out for C, H and N at the Microanalytical Centre of Cairo University. All reagents were purchased from the Aldrich chemical company.

Method for synthesis of 2-mercapto-*1H*-benzimidazole (2)

A mixture of *o*-phenylenediamine **1** (2.16 g, 0.02 mol), carbon disulfide (2.25 g, 0.03 mol), and KOH (1.10 g, 0.02 mol) were heated under reflux for 6 hours. On cooling, the reaction mixture was neutralized with conc. hydrochloric acid and the separated solid was filtered, dried and crystallized from aqueous ethanol Yield, 92%; m.p. 297 ^{o}C (Lit. m.p. 300 ^{o}C).

Method for synthesis of 2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazole (3)

A mixture of 2-mercaptobenzimidazole **2** (1.5 g, 0.01mol) in dimethylformamide (30 ml), dry potassium carbonate (1.51 g, 0.01mol) and 5-chloro N-phenyltetrazole (1.4 g, 0.01mol) was stirred for 18 hours. The reaction mixture was then treated with ice cold water and the separated solid was filtered, dried and crystallized from aqueous ethanol.

Yield, 93%; m.p. 148 ^{o}C; IR (KBr) ν_{max}/cm^{-1}; 3320 (NH).^{1}H-NMR δ 13.03 (s, 1H, NH, D_2O exchangeable), 7.73 (m, 2H, Ar-H), 7.62 (m, 2H, Ar-H), 7.54 (m, 3H, Ar-H), 7.24 (m, 2H, Ar-H). MS m/z (%); 295.90 (M^+ +2, 1.18), 294.90 (M^+ +1, 3.69), 293.90 (M^+, 18.51), 292.95 (M^+-1, 0.09), 217.95 (0.30), 149.95 (10.46), 148.95 (100), 146.05 (0.07). Anal. Calcd. for $C_{14}H_{10}N_6S$ (%): C, 57.13; H, 3.42; N, 28.55. Found: C, 57.45; H, 3.44; N, 28.76.

Method for synthesis of ethyl-3-(2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazol-1-yl) propanoate (4)

To a solution of **3** (2.94 g, 0.01mol) in tetrahydrofuran (30 ml), NaH (0.24 g, 0.01mol) was added under nitrogen pressure and stirred for 2 hours. Ethyl bromopropionate (2.71 g, 0.01mol) was then added and the reaction mixture was stirred for 24 hours. Cold ice water (3 ml) was added, followed by evaporation under reduced pressure to about 5 ml. The reaction mixture was extracted 3 times with ethyl acetate. Ethyl acetate extract was washed with brine solution (30 ml), filtered over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dried and crystallized from aqueous ethanol.

Yield, 87%, m.p. 162 ^{o}C; IR (KBr) ν_{max}/cm^{-1}; 1744 (C=O), 1590 (C=N). ^{1}H-NMR δ 7.80 (d, 2H, Ar-H), 7.60 (m, 3H, Ar-H), 7.40 (d, 2H, Ar-H), 7.20 (m, 2H, Ar-H), 4.05 (q, 2H, $OC\underline{H_2}CH_3$), 3.30 (m, 4H, CH_2CH_2), 1.71 (t, 3H, $OCH_2C\underline{H_3}$). MS m/z (%); 395.10 (M^++1, 33.53), 394.10 (M^+, 17.22), 393.10 (M^+-1, 1.51), 250 (46.53), 246 (17.82), 179 (15.11), 160.05 (100). Anal. Calcd. for $C_{19}H_{18}N_6O_2S$ (%): C, 57.85; H, 4.60; N, 21.31. Found: C, 57.43; H, 4.20; N, 21.72.

Method for synthesis of 3-(2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazol-1-yl)propane hydrazide (5)

Hydrazine hydrate (98%) (0.64 g, 0.02 mol) was added to a solution of the ester **4** (3.94 g, 0.01 mol) in absolute ethanol (40 ml). The reaction mixture was heated under reflux for 2 hours, cooled and the separated solid was filtered, dried and crystallized from ethanol.

Yield, 85%; m.p. 185 ^{o}C; IR (KBr) ν_{max}/cm^{-1}; 3320 (NH_2), 3295 (NH), 1685 (C=O), 1590 (C=N). ^{1}H-NMR δ 12.48 (br. s, 1H, NH, D_2O exchangeable), 9.18 (s, 2H, NH_2, D_2O exchange), 7.8 (m, 1H, Ar-H), 7.63 (m, 1H, Ar-H), 7.60 (d, 1H, Ar-H), 7.42 (d, 1H, Ar-H), 7.3 (m, 1H, Ar-H), 7.22-7.12 (m, 2H, Ar-H), 7.00 (m, 1H, Ar-H), 6.7 (m, 1H, Ar-H), 3.29 (br. s, 4H, CH_2CH_2). MS m/z (%); 382.20 (M^++2, 3.03), 381.15 (M^++1, 3.57), 380.15 (M^+, 0.66), 379.15 (M^+-1, 2.21), 336.10 (1.15), 304.05 (0.81), 274.05 (0.66), 57 (100). Anal. Calcd. for $C_{17}H_{16}N_8OS$ (%): C, 53.67; H, 4.24; N, 29.45. Found: C, 53.40; H, 3.98; N, 29.74.

Method for synthesis of 5-(2-(2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazol-1-yl)ethyl)-1,3,4-oxadiazole-2-thiol (6)

To a mixture of the acid hydrazide **5** (3.8 g, 0.01mol) and KOH (0.56 g, 0.01mol) in 95% ethanol (50 ml), carbon disulfide (0.84 g, 0.01mol) was added. The reaction mixture was heated under reflux for 12 hours and the solvent was evaporated under reduced pressure. The obtained residue was then dissolved in water, neutralized with concentrated hydrochloric acid and the separated solid was filtered, dried and crystallized from aqueous ethanol.

Yield: 80%; m.p. 214 ^{o}C; IR (KBr) ν_{max}/cm^{-1}; 1600 (C=N). ^{1}H-NMR δ12.47 (br. s, 1H, SH), 7.8-7.65 (m, 2H, Ar-H), 7.50-7.25 (m, 2H, Ar-H), 7.20-7.00 (m, 3H, Ar-H), 6.90 (m, 1H, Ar-H), 6.75-6.50 (m, 1H, Ar-H), 3.49 (m, 4H, CH_2CH_2). MS m/z (%); 423 (M^++1, 3.31), 422 (M^+, 16.57), 389 (23.80), 232 922.89), 178 99.04), 76.95 (100). Anal. Calcd. for $C_{18}H_{14}N_8OS_2$ (%): C, 51.17; H, 3.34; N, 26.52. Found: C, 51.58; H, 3.74; N, 26.42.

Method for synthesis of 4-phenyl-5-(2-(2-(1-phenyl-*1H*-tetrazol-5-ylthio)-*1H*-benzimidazol-1-yl)ethyl)-*4H*-1,2,4-triazole-3-thiol (7)

To a mixture of the acid hydrazide **5** (3.8 g, 0.01mol) and KOH (0.56 g, 0.01mol) in 95% ethanol (50 ml), phenylisothiocyanate (1.35 g, 0.01 mol) was added. The reaction mixture was heated under reflux for 12 hours and the solvent was evaporated under reduced pressure. The obtained residue was then dissolved in water, neutralized with concentrated hydrochloric acid and the separated solid was filtered, dried and crystallized from aqueous ethanol.

Yield: 78%; m.p. 198 ^{o}C; IR (KBr) ν_{max}/cm^{-1}; 1580 (C=N). ^{1}H-NMR δ 11.00 (s, 1H, SH), 7.72-7.14 (m, 14H, Ar-H), 3.43 (m, 4H, CH_2CH_2). MS m/z (%); 498.10 (M^++1, 37.50), 497.10 (M^+, 44.74), 496.10 (M^+-1, 48.68), 354.10 (8.55), 345.10 (7.24), 313.10 (1.32), 55 (100). Anal. Calcd. for $C_{24}H_{19}N_9S_2$ (%): C, 57.93; H, 3.85; N, 25.33. Found: C, 57.50; H, 3.05; N, 25.73.

4.2 Molecular Docking methodology

The primary molecular modeling study of the newly synthesized compounds was performed by submitting their structures to the free online site Pharm Mapper. Docking calculations were then carried out using the online site DockingServer which perform docking using AutoDock 4.0.

4.3. Biological Activities

4.3.1. Cytotoxic activity

The cytotoxic activity of the newly synthesized compounds was determined using the *in vitro* MTT assay (Mosmann 1983). The cell lines were obtained from American Type Culture Collection (ATCC). The cells were cultivated at 37°C and 10% CO_2 in RPMI-1640 (Lonza, 12-702F) medium supplemented with 10% fetal bovine serum (FBS, Lonza, Cat. No.14-801E), 100 IU/ml penicillin and 100 µg/ml streptomycin (Lonza, 17-602E).

Inhibition of proliferation was measured in 96-well plate. 60 µL of serial dilutions of the test compounds dissolved in 0.05% DMSO were given to 120 µL of the suspended cells (50,000/mL) in wells of 96-well plates. The metabolic activity of the cells was measured by MTT assay after 5 days of incubation at 37^{0}C and 10% CO_2. 20µl MTT (5 mg/ml PBS, SERVA, Cat. No. 20395.01) was added to each well and incubated for 4 hours at 37°C and 10% CO_2. After that the medium was removed, the formed purple formazan particles were dissolved by adding 100 µl Isopropanol/HCl solution. The plates were then incubated for 15 min on shaker (600 r.p.m/ min) to dissolve the formazan crystals. The intensity of the purple color was measured at λ 540 nm by microplate reader (ELx800 Absorbance Microplate Reader, BioTek) against DMSO as a negative control. The cytotoxic activity of the tested compounds was expressed as the concentration that caused 50% growth inhibition (IC_{50}) compared to the untreated cells (DMSO without the tested compounds).

4.3.2. Microbiological activity

In vitro antimicrobial activity was carried out using disc diffusion assay (Collins 1964, Lorian 1980). Whatman No. 1 filter paper disc of 5 mm diameter were sterilized by autoclaving for 15 min at 121 ^{o}C. The sterile discs were impregnated with the test compounds (500µg/ disc). The agar plates were inoculated with standard incolum (10^5 cells/ mL broth) of the test organisms (local strains) namely, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* The impregnated discs were placed on the agar plate medium, and the plates were incubated at 5 ^{o}C for 1 hour to permit good diffusion and then transferred to an incubator at 37 ^{o}C for 24 hours. The diameter of the inhibition zone was measured using a caliber; to the nearest mm. Gentamycin was used as a standard. The test compounds were subjected to a quantitative assay in order to determine their minimum inhibitory concentrations (MICs) using the two-fold serial broth dilution assay (Stokes and Ridgway, 1980, Wenson *et al.*, 1982). Standardized bacterial inocula were prepared by touching the top of four or five colonies of single type and inoculating them into a tube containing 5 mL of Mueller-Hinton broth (Difco) at pH 7.3. Incubation of these micro-organisms suspensions were carried out at 35 ^{o}C until a visible turbidity was obtained. Finally, the culture was diluted so that, after inoculation, each microplate well had an inoculm size of $5X10^5$ colony forming units/mL. Antibacterial assays were performed in Mueller-Hinton (Difco) at pH 7.3. Gentamycin was used as standard drug. All the tested compounds were dissolved in DMSO. Further dilution of the test compounds and the standard drug in the test medium were furnished at the required quantities of the broth used. The concentration range was 32-0.015 µg/ml for Gentamycin and 128-1 µg/ml for the test compounds. After inclusion of 100 µg/ml of the broth containing the standard drug or the test compound, 100 µg/ml of bacterial suspension were inoculated into microplate wells. After incubation for 16-20 hours at 35 ^{o}C, the well containing the lowest concentration of the standard drug or the test compound that inhibit micro-organism growth as detected by the unaided eye, was recorded to represent the MIC expressed in µg/ml.

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