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Consolidated antimicrobial and anticancer activities through newly synthesized novel series of pyrazoles bearing indazolylthiazole moiety: characterization and molecular docking

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Abstract

A new series of pyrazoles have been synthesized via 2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazol-4(5H)-one(**2**), which on treatment with phosphorus pentachloride and phosphoryl chloride afforded 4-chlorothiazole derivative(**3**). Reaction of **3** with hydrazine hydrate in boiling gave 4- hydrazineylthiazole derivative (**4**). Heterocyclization of **4** with aromatic aldehydes and active methylene compounds afforded the pyrazole derivatives (**5-8**).The synthesized pyrazoles (**5–8**) and their precursor (**1–4**) were evaluated for antimicrobial activities. All prepared compounds revealed abroad-spectrum antimicrobial activity with maximum inhibition activity against *Streptococcus mutans*. Compound **3** revealed the most potent antibiofilm inhibition activity against the three used pathogens. Additionally, all compounds (**1-8**) were also tested for cytotoxic activity against hepatoma, and colon carcinoma cell lines. Compounds **3**, **5** and **7** displayed good to excellent activity against all tested tumor cells with IC50 values ranged from 6.13 to 23.85 µg/ml. On the other hand, all compounds were evaluated for cytotoxic activity on normal human melanocytes cell lines and found to be signified its high selectivity toward cancer cells than normal cells. Moreover, the molecular modeling study was carried out using (MOE 2014) software. The computational studies are confirming the results in biological activity.

Keywords: Microwave-assisted synthesis; Indazolylthiazolodinone; Hydrazineyl Indazolylthiazole; Pyrazoles; Antimicrobial; Anti-cancer; Molecular docking.

1. Introduction

The rapid evolutionary spread of antibiotic-multiresistant pathogens represents an emerged challenge for human health care [1]. Several mechanisms are developed by microorganisms to resist applied antimicrobial agents, including inactivation-enzymes production, efflux pumps, modifying the drug targets, and microbial-biofilm formation [2]. Microbial pathogens in a biofilm are less susceptible to antimicrobial agents and can evade the host immune system [3]. A dual strategy has been applied to deal with this issue by developing novel antibiotics and/or modifying existing antibiotics [4]. On the other hand, cancer represents another pressing challenge for human beings coming next to cardiovascular diseases as the second causative agent of human mortality [5]. Genetically impaired regulation for cell proliferation and differentiation distinguishes cancer cells from normal ones [6]. A variety of methods have been applied for cancer cell suppression and treatment including surgical removing, radiation, and chemotherapy treatment. To date, none of the applied treatments satisfy the requirements for selectivity and high efficacy toward cancer cells and post-treatment side-effects are numerous and serious. Therefore, the continuous research for developing novel structures that could be exhibit active, selective, and less toxic anticancer and/or antimicrobial agents is a pressing necessity [7,8].

Ever since the earliest isolation of pyrazole in *Houttuynia cordata* extract, its structure represents the main core for many applied drugs [9,10], moreover, its derivatives revealed numerous potent biological activities [9,10]. Indazole and its structurally diverse

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derivatives have played a vital role. Diversely substituted indazole derivatives with different functional groups have attracted much attention in the past, as well as in recent years, because of their different kinds of biological properties, such as anti-inflammatory, antibacterial, anti-HIV, anti-arrhythmic, antifungal, and antitumor [11–14]. On the other hand, thiazole is the most common heterocyclic compound in heterocyclic chemistry and drug design. The thiazole ring is present in vitamin B1 (Thiamine) and penicillin [15]. Thiazole derivatives have a wide range of medicinal and biological properties, including antibacterial, antifungal [16], anti-inflammatory [17], antimalarial [18], and anti-HIV activities [19].

All of these encouraged us to integrate these two heterocycles for synthesizing a novel series of pyrazole derivatives incorporating indazolylthiazole moiety and evaluate the newly synthesized compounds which may be exhibit synergistic antimicrobial and anticancer effects.

2. MATERIALS AND METHODS

2.1. Chemistry

All melting points are uncorrected and were determined on Gallenkamp electric melting point apparatus. All products were characterized by IR, 1H-NMR, 13C-NMR, and Mass spectral data. The 1H and 13C-NMR spectra were recorded by using (CDCl3/ DMSO-d6) as solvents on Brucker 300MHZ spectrometer with tetramethylsilane (TMS) as an internal reference.

General procedure

2.1.1. Synthesis of 3-phenyl-1,3,4,5,6,7-hexahydro-2H-indazole-2-carbothioamide(1).

A mixture of cyclohexanone (0.01mol, 1mL), benzaldehyde (0.01mol, 1mL) in sodium hydroxide20 %(10mL) were added in Pyrex flask. The mixture was heated under solvent free and microwave irradiation conditions for 60 sec, thiosemicarbazide (0.01mol, 1gm) was added. The mixture was heated for the time needed to complete the reaction (Monitored by TCL). The reaction mixture cooled to room-temp, neutralized with 20 cm^3 4N HCl. The pure product separated and recrystallized from mixture of ethanol and water in 1:1 ratio to give the required product (**1**) as yellow crystals, in 88% yield, m.p.120-122^{0}C: Requires: C, 64. 83; H, 6.61; N, 16.20; S, 12.36. Found: C, 64. 64; H, 6.41; N, 16.01; S, 12.23. IR (KBr): ν (cm^{-1}) 1188 (C=S); 1507(C=C); 3379, 3194 (NH2). ^{1}H-NMR (300 MHz, DMSO-d6) δ:1.50-2.48 (8H,m,4CH2);5.20(1H,s,CH); 8.15(1H,s,br, NH); 11.40(2H,s, NH2CS↔ NH-SH);7.27-7.45 (5H , m , 5H-Ar).

Synthesis of 2-(3-phenyl-1,3,4,5,6,7-hexahydro-2H-indazol-2-yl)thiazol-4(5H)-one (2).

A mixture of *3-phenyl-1,3,4,5,6,7-hexahydro-2H-indazole-2-carbothioamide(**1**)*(0.01mol, 2.6gm), and α-chloro acetic acid (0.01mol, 0.9gm) was heated under reflux in dimethyl formamide (25 mL) for 12 hrs., cooled to room-temp, poured onto ice water. The yellow precipitate formed after cooling was filtered off, washed with water, dried and recrystallized from benzene to afford the required product (**2**) as yellow powder, in 52% yield, m. p 100 -103 ^{0}C. Requires: C, 64. 19; H, 5.72; N, 14.04; S, 10.71. Found: C, 64.01; H, 5.50; N, 13.98; S, 10.53. IR (KBr): ν (cm^{-1}) 1598 (C=C), 1627 (C=N), 1710(C=O), 3228(NH). ^{1}H-NMR(300MHz,DMSO-d6):*δ*1.60-2.49(8H,m,4CH2); 3.89(2H,s,SCH2);4.25(1H,s,CH);7.11-7.76(5H,m, Ar-H);8.41(1H,s,NH). ^{13}C-NMR (100 MHz, DMSO-d6): *δ* 21.02, 22.44, 27.09, 32.80 (4CH2); 38.63 (SCH2), 109.08 114.10,125.08- 137.72((aromatic >C=C <), 156.18, 168.11, 174.18 (hetero aromatic>N-C=N <, >N-C=O <).

2.1.2. Synthesis of 4-chloro-2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazole (3).

A mixture of **2** (0.01mol, 3gm), phosphoryl chloride (0.01mol, 1.5mL) and phosphorus pentachloride (0.01mol, 2gm) was heated under reflux on water bath for 4 hrs. , cooled to room-temp, poured onto ice water and HCl left overnight. The solid that separated filtered off, washed with water and recrystallized from ethanol to afford the required product (**3)** as brown powder in 62%yield, m.p140-142^{0}C. Requires: C, 60.46; H, 5.07; N,13.22; S,10.09 ;Cl,11.15. Found:C,60.31;H,5.01;N,13.14;S,10.00;Cl,11.02. IR(KBr):ν(cm^{-1})1600(C=C);1627(C=N); 3190(NH); 752(C-Cl).^{1}H-NMR(300MHz,DMSO-d6):*δ*1.68-2.49(8H,m,4CH2);5.07(1H,s,,br,CH); 6.35(1H,s,CH) ;6.92-7.72(6H,m,Ar-H);10.0(1H,s,NH).^{13}C-NMR (100MHz,DMSO-d6):*δ*21,22,26,27(4CH2); 69(CH); 126,127, 128,129, 130,131,134, 138,145,156 (aromatic >C=C < and hetero aromatic >N-C-Cl <, >N-C=N).

2.1.3. Synthesis of 4-hydrazineyl-2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazole (4).

To ethanolic solution of 2-(4-chlorothiazol-2-yl)-3-phenyl-2, 3, 4, 5, 6, 7-hexahydro-1H-indazole **3** (0.01mol,3gm), hydrazine hydrate (0.01mol,0.5mL) was added. The reaction mixture was heated under reflux for 6 hrs. , the solid obtained after evaporation of solvent was collected and recrystallized from ethanol to afford the required product (**4**) as red powder in 39% yield, m. p 130 -132 ^{0}C. Requires: C, 61. 31; H, 6.11; N, 22.34; S, 10.23. Found: C, 61.19; H, 6.01; N, 22.17; S, 10.14. IR(KBr):ν(cm^{-}

^{1})1507(C=C);3330,3200;3583(NHNH2).^{1}H-NMR(300MHz,DMSO-d6):δ1.63-2.21(8H,m,4 CH2);5.4(1H,s,CH);[4.45(2H,d,NH2);8.55(H,s,br,N H)exchangeable withD2O];7.15-7.67(5H,m,Ar-H) .

2.1.4. Synthesis of ethyl 3-methyl-5-phenyl-1-(2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazol-4-yl)-2,3-dihydro-1H-pyrazole-4-carboxylate (5).

A mixture of ethyl acetoacetate (0.01mol,1.3mL) and benzaldehyde (0.01mol,1mL) in ethanol (50 mL) with catalytic amount of piperidine (0.5mL) was heated under reflux for 2 hrs., compound **4** (0.01mol,3gm) was added and the reaction mixture was heated under reflux for another 12 hrs., cooled to room-temp, acidified by HCl until precipitate. The solid formed was filtered off, washed with water and purified by recrystallization from benzene to afford the required product (**5**) as brown powder in 63% yield, m. p 103-105 ^{0}C. Requires: C, 65. 96; H, 6.77; N, 14.24; S, 6.52; Found:C,65.81;H,6.63;N,14.11; S,6.30. IR(KBr):ν(cm^{-1}) 1449(CH3);1601 (C=C) ; 1620(C=N);1720 (CO);3215(NH).^{1}H-NMR(300MHz,DMSO-d6):δ1.122(3H,t,CH2CH3) ;1.55-1.97 (8H,m,4CH2) ;2.13(3H,s, CH3) ;4.09 (2H,q,CH2CH3) ;4.15 (1H,s,H3-pyrazole); 6.97(1H,s,CH-thiazole);7.25-7.94(10H,m,Ar-H);10.00(1H,s,NH-pyrazole) with D2Oexchangeable .^{13}C-NMR (100MHz,DMSO-d6) :δ13.6;14.7(2CH3); 21.25,22.04,25.84, 26.60 (4CH2);60.7(CH2CH3); 73.9; 77.8; 102.3, 109.1, 112.3,125.4,126.6,127.3, 128.3,142.0,143.5, 144.0, 151.20(aromatic>C=C<); 164.3,166.7 (heteroaromatic> C=N <, > C=O <).

2.1.5. Synthesis of ethyl 3-amino-5-phenyl-1-(2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazol-4-yl)-2,3-dihydro-1H-pyrazole-4-carboxylate (6).

A mixture of ethyl cyanoacetate (0.01mol,1mL) and benzaldehyde (0.01mol,1mL) in ethanol(50 mL) with catalytic amount of piperidine(0.5mL) was heated under reflux for 2 hrs., compound **4** (0.01mol,3gm) was added. The reaction mixture was heated under reflux for another 12 hrs. , cooled to room-temp, acidified by HCl until precipitate. The solid formed was filtered off, washed with water and purified by recrystallization from benzene to afford the product (**6**) as brown powder in 33% yield, m. p 90 -93 ^{0}C. Requires: C, 64.59; H, 6.97; N, 16.14; S, 6.16. Found: C, 64.42; H, 6.79; N, 16.09; S, 6.02. IR(KBr):ν(cm^{-1})1367(CH3);1493 (C=C) ;1600 (C=N); 1737 (CO);3200,3350(NH2) .^{1}H-NMR (300MHz,DMSO-d6):δ1.03(3H,t,CH2CH3);1.13-2.23 (8H,m,4CH2); 2.87(1H,d,H4-Pyrazole);4.45 (2H,q,CH2CH3);4.80(1H,d,H5-pyrazole);6.82 (2H,s,NH2) exchangeable with D2O; 7.22-7.92 (10H,m,Ar-); 8.32(1H,s, NH-indazole).

2.1.6. 1-(3-methyl-5-phenyl-1-(2-(3-phenyl-1, 3, 4, 5, 6, 7-hexahydro-2H-indazol-2-yl)thiazol-4-yl)-1H-pyrazol-4-yl)ethan-1-one (7).

A mixture of acetyl acetone (0.01mol,1mL) and benzaldehyde (0.01mol,1mL) in 50 ml ethanol containing catalytic amount of piperidine(0.5mL) was heated under reflux for 2 hrs., compound **4** (0.01mol,3gm) was added, the reaction mixture was heated under reflux for another 12 hrs., cooled to room-temp, acidified by HCl until precipitate. The solid formed was filtered off, washed with water and purified by recrystallization from petroleum ether 60-80 ^{0}C to afford the product (**7**) as brown powder in 49% yield, m. p 100 -102^{0}C.Requires:C, 69.54; H, 6.04;N,14.48;S,6.63.Found: C,69.42;H,6.00;N,14.40; S,6.51.IR(KBr):ν(cm^{-1})1602(C=C);1658(C=N); 1707 (CO);3358(NH).^{1}H-NMR(300MHz,DMSO-d6): δ1.56-197(8H,m,4CH2);1.22(3H,s,CH3); 2.33(3H,s,br,COCH3);3.16(1H,s,CH-pyrazole);6.20(1H,s,CH-thiazole);7.19-7.66(10H,m,Ar-H);10.0(1H,s,NH-pyrazole).^{13}C-NMR(100MHz,DMSO-d6):δ17.64(CH3); 30.03(COCH3);56.32;62.12;76.82;110.6-145.03 (aromatic>C=C<);156.70;164.8;181.02(hetero aromatic> C=N <, > C=O <).

2.1.7. Synthesis of 5-amino-3-phenyl-2-(2-(3-phenyl-3,3a,4,5,6,7-hexahydro-2H-indazol-2-yl)thiazol-4-yl)-2,3-dihydro-1H-pyrazole-4-carbonitrile (8).

A mixture of malononitrile (0.01mol,0.7mL) and benzaldehyde (0.01mol,1mL) in 50 ml ethanol with a catalytic amount of piperidine(0.5mL) was heated under reflux for 2 hrs., compound **4** (0.01mol,3gm) was added, the reaction mixture was heated under reflux for another 12 hrs., cooled to room temp, acidified by HCl until precipitate. The solid formed was filtered off, washed with water, and purified by recrystallization from petroleum ether 60-80 ^{0}C to give the product **8** as a brown powder in 49% yield, m. p 93 -95 ^{0}C. Requires: C, 66. 78; H, 5.39; N, 20.97; S, 6.86. Found: C, 66. 69; H, 5.27; N, 20.83; S, 6.79. IR(KBr):ν(cm^{-1})1587(C=C); 1625(C=N); 2215 (CN); 3328-3212(NH2). ^{1}H-NMR(300MHz,DMSO-d6): δ1.20- 2.49 (8H, m,4 CH2); 2.85,3.84(2H,d,2CH-pyrazole); 6.96(2H,br,NH2) with D2O exchangeable;7.30-7.54 (10H,m,Ar-H).

2.2. Biological activity

2.2.1. Antimicrobial efficacy of the prepared compounds

The antimicrobial activity of the synthesized compounds (**1-8**) was evaluated against three representative human pathogens including Gram-positive bacterium (G +ve) *Streptococcus mutans* ATCC 25175, Gram-negative bacterium (G–ve) *Pseudomonas aeruginosa* ATCC 27853, and

unicellular fungi_*Candida albicans*_ATCC 10231 using microdilution assay as following: each compound was tested in five different concentrations (5-80 µg) against the three pathogenic organisms in flat-bottom 96 well tissue culture plate. The pre-inoculation culture was prepared by overnight cultivation of the three organisms on a nutrient broth medium at 37°C. Each well was inoculated with 100 µl of the diluted tested organism (10^6 CFU/ml), and specific compound concentration to a final volume of 200 µl. The microtiter plate was incubated overnight at 37°C and measured at 600 nm with a microplate reader. Ampicillin, ciprofloxacin and clotrimazole were included in the experiments as reference antimicrobial drugs. Results were expressed as minimum inhibitory concentration (MIC) indicating the lowest concentration that completely eradicated cells growth.

2.2.2. Microbial-biofilm inhibition assay

Inhibition of microbial-biofilm formation was assayed for prepared compound on the above mentioned human pathogens using tissue culture plate technique (TCP) according to [29] with simple modifications as following: tested organisms were cultivated overnight at 37°C on LB broth medium. A diluted 200 µl of these cultures (10^6 CFU/ml) were inoculated in triplicates into 96 well tissue culture plates and incubated overnight at 37°C to allow microbial-biofilm to be formed. Upon overnight incubation, the free-floating microbial cells were decanted and each well was washed using PBS buffer pH 7.1 three times. A fresh LB medium was added to each well with 25 µl (25 µg/ml final concentration) of the tested compounds and incubated overnight at 37°C. For the untreated group (control) only fresh LB medium was added without any compound. After incubation, the free-floating cells were decanted again and the plate was washed three times with PBS buffers. Adherent cells-biofilm was stained for 5 min with crystal violet solution (0.1% w/v). After washing the excess crystal violet solution, the stained biofilm was solubilized using 30% (v/v) glacial acetic acid, where the resulting color was measured at 590 nm and compared to untreated cells (control).

2.2.3. *Cytotoxicity against normal cells*

To evaluate the cytotoxicity of the newly synthesized compounds at different concentrations on normal cells, the hydrogen acceptor method colorimetric MTT (3-[4, 5-Dimethylthiazol]-2, 5-Diphenyltetrazolium bromide) method was carried out [24,25]. The yellow color of tetrazolium salt is reduced by viable cells via mitochondrial dehydrogenases to produce insoluble formazan crystals, which were converted to purple by the addition of dimethyl sulfoxide (DMSO). HFB-4 (normal human melanocytes) cells were seeded into a sterile 96-well microplate and cultured overnight in a complete DMEM medium (Lonza, USA) as 1.0×10^4 cells per well. Different concentrations of the tested compounds (2, 4, 8, 16, 32, and 64 µg/ml) were added to HFB-4 cells in triplicates. After incubation at 37°C for 48h in a 5% CO_2 incubator, the cells were washed 3 times with fresh media to remove dead cells and debris, then a solution of 0.5 mg/ml MTT (Sigma-Aldrich) was added to the cells. The plates were further incubated at 37°C for 2-5 h, then the MTT solution was removed and substituted with 200 µl of DMSO. The absorbance of viable cells was measured at 570 nm using a microplate reader (BMG LabTech, Germany), and the half-maximal inhibitory concentration (IC50) values of the tested derivatives were determined using the GraphPad Prism 6.0 software.

2.2.3. The anticancer efficacy of the newly prepared compounds

The cytotoxic effect of the newly synthesized compounds against Caco-2 (human colon carcinoma) and HepG-2 (human hepatoma) cell lines was evaluated by the colorimetric MTT method as described above. Caco-2 and HepG-2 cells (1.0×10^4 cells/well) were seeded into sterile 96 well tissue culture plates and incubated for 24 h before adding the tested compounds. Various concentrations of the tested derivatives (2, 4, 8, 16, 32, and 64 µg/ml) were added to cells in triplicates. After cells incubation for 48 h in a 5% CO_2 incubator, the MTT assay was carried out as described above. The absorbance of viable cells was measured at 570 nm using an ELISA plate reader. The percentage of relative viability of tumor cells for each concentration of the tested derivative individually was calculated according to the following equation:

The relative cell viability (%) = [Xt-Xb/Xc-Xb] × 100

Where: Xt is the absorbance of the test derivative, Xb is the absorbance of blank and Xc is the absorbance of control.

The effective antitumor effect of the tested derivatives was estimated by calculating the IC_{50} value which means 50% cytotoxicity or the concentration of the derivative causing 50% cell death was determined by the software of GraphPad InStat 6.0 using data obtained from the above-mentioned equation (percentage of relative cell viability). The selectivity index (SI) values defined as the ratio of the IC50 on normal human cells (HFB-4) versus the IC_{50} values of each tumor cell line were also calculated [24,30]. In addition, the effect of the highly potent derivatives (3, 5, and 7) on the morphology of all tested tumor cells was investigated at different concentrations (4-16 µg/ml) through phase-contrast microscopy (Olympus, Germany) in comparison with untreated cells as a negative reference.

2.2.4. Effect of the potent derivatives on gene expression

The effect of the potent prepared derivatives on the expression of some tumor genes was estimated through quantitate detection of tumor suppressor gene (p53), oncogene (Bcl-2), vascular endothelial growth factor gene (VEGF), Matrix Metalloproteinase gene (MMP-9), and beta-catenin protein gene (β-catenin) in human Caco-2 and HepG-2 cell lines. After treating of cells by IC_{50} concentrations for compounds 3, 5, and 7 for 2 days, total RNAs were extracted using the protocol of Gene JET RNA Purification Kit (Thermo Scientific, USA). CDNA synthesis was carried out by the cDNA Synthesis Kit (Thermo Scientific, USA), and real-time PCR was performed by a master mix of SYBR green kit. Specific primers (Forward/Reverse) as follow: 5′-TAACAGTTCCTGCATGGGCGGC-3′; 5′-AGGACAGGCACAAACACGCACC-3′ for p53 gene, 5′-TCCGATCAGGAAGGCTAGAGTT-3′/5′-TCGGTCTCCTAAAAGCAGGC-3′ for Bcl-2: 5'-GGCTTTACTGCTGTACCTCC-3'/ 5'-CAAATGCTTTCTCCGCTCT-3' for VEGF gene, 5'-CTGCGTATTTCCATTCATC-3'/ 5'-CCTTGGGTCAGGTTTAGAG-3' for MMP-9 gene and 5'-CATATGCGGCTGCTGTTCTA-3'/ 5'-CCGAAAGCCGTTTCTTGTAG-3' for β-catenin gene. The equation of 2-ΔΔCT was utilized to determine the alteration of each gene expression for Caco-2 and HepG-2 cells after and before treatment.

2.2.5. Molecular docking analysis

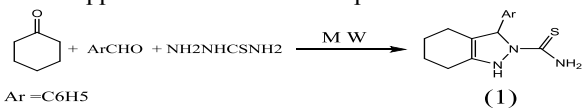
The molecular modeling study was carried out using Molecular Operating Environment (MOE 2014) software. The three-dimensional structures and conformations of the proteins were downloaded from the Protein Data Bank (PDB) website. The molecules under study were constructed in MOE using the builder module, prepared, and then collected in a database. The compounds were prepared by the addition of hydrogens using the option "Protonate 3D" and energy minimization (using Force Field MMFF94x). At the same time, the downloaded protein was prepared by deleting the repeated chains and water molecules. Hydrogens were also added to the atom of the receptor and the partial charges were calculated. Then, the protein energy was minimized. Lastly, the pocket was isolated. Validation of docking protocol was confirmed by re-docking of the downloaded ligand into its pocket. The obtained root mean standard deviations (RMSD) were found to be less than 1.5 Å. It was reported that values less than 1.5 or 2 Å were indicators of a successful and reliable docking protocol. MOE was used to calculate the binding energies of the interactions between the ligands and the pocket. Scoring was calculated in Kcal/mol and was determined using alpha HB as a scoring function [31,32].

3. RESULTS AND DISCUSSION

3.1. Chemistry

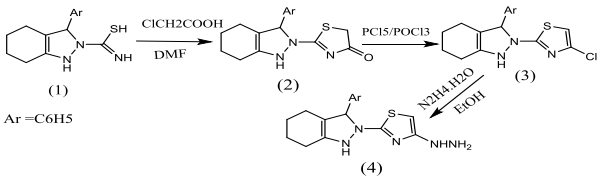
The starting 3-phenyl-1,3,4,5,6,7-hexahydro-2H-indazole-2-carbothioamide (**1**) was obtained in a good yield via one-pot three-component condensation of benzaldehyde, cyclohexanone, and thiosemicarbazide under basic and microwave irradiation reaction conditions [Scheme 1].

The IR spectrum of compound **1** showed an absorption bands at 1188, 3194, 3379cm-1 attributed to υC=S and υNH2 and the 1H-NMR spectrum, showed a multiplet signal at δ1.50-2.48ppm due to cyclohexene protons, one broad signal at δ 11.40ppm due to CSNH2 (NH-SH) protons, multiplet signal at δ 7.27-7.45ppm due to Ar-H five protons and, one broad signal in the region at δ8.15ppm due to indazole NH proton.

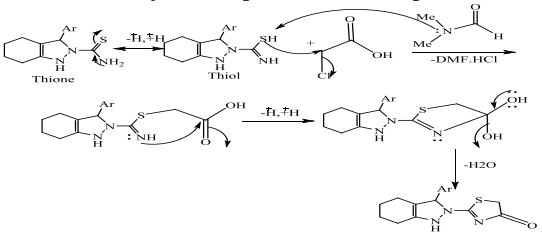


Scheme 1. Synthesis of the target compound 1 via one-pot three-component reaction.

In boiling DMF [20] compound 1 was reacted with α-chloroacetic acid to afford 2-(3-phenyl 1,3,4,5,6,7-hexahydro-2H-indazol-2-yl)thiazol-4(5H)-one (2).The proposed structure was proved by elemental analysis and spectral data. The IR spectrum showed absorption band at 1710 cm-1 and in 1H-NMR spectrum one singlet appeared at δ3.89,this is indicating the presence of carbonyl in IR spectrum and methylene group in NMR proton with the lack of υNH2 and υC=S due to cyclization. In addition, the 13C- NMR spectrum revealed characteristic signals at δ38.63, 156.18, 168.11 and 174.18 ppm for SCH2 C=N and C=O for thiazole moiety confirmed its structure. Treatment of derivative **2** with phosphorus pentachloride and phosphoryl chloride afforded 4-chlorothiazole derivative (**3**) (Scheme 2). The structure of **3** was supported by the 1H-NMR spectrum which indicated that the most characteristic signal of compound 2 corresponding to thiazolidine methylene protons has completely disappeared, besides the lack of the carbonyl band in (IR) spectrum. While, the 13C- NMR spectrum showed characteristic bands at 134 and 156ppm attributed to hetero-aromatic > C-Cl < and > C=N with the disappearance of Carbonyl carbon atom. Reaction of **3** with hydrazine hydrate[21] in boiling ethanol, afforded 4-hydrazineylthiazole derivative (**4)**, the compound, correctly analyzed for its molecular formula, showed in the IR spectrum strong bands at 3330, 3200, 3583 cm-1 due to υNHNH2 with the disappearance of υ C-CL. The 1H-NMR spectrum assigned the presence of one singlet at δ 5.4 ppm of thiazole ring proton and two signals appeared at δ 4.35 and 8.55 ppm owing to NHNH2 protons with D2Oexchangeable.

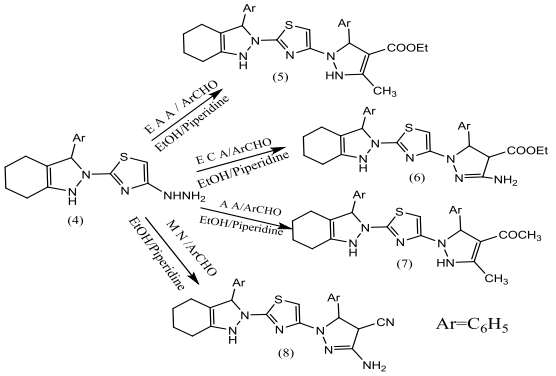


Scheme 2. Synthetic protocol of compounds **2**–**4**.

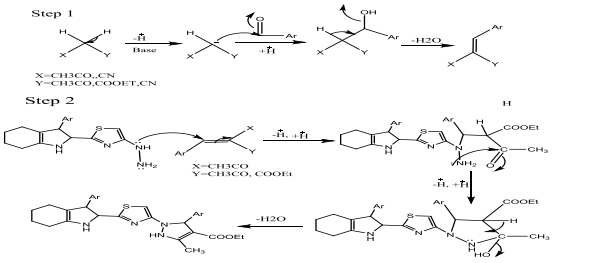


Mechanism pathway for conversion of indazole-2- carbothioamide into indazolylthiazolidin-4one

With the selection of appropriate cyclizing agents, a series ensemble of five-membered diaza heterocyclic system has been synthesized, where product **4** reacted with three carbon donor compounds namely (ethyl acetoacetate, ethyl cyanoacetate, acetylacetone, and malononitrile) and benzaldehyde through one pot multicomponent reaction in boiling ethanol containing catalytic amount of piperidine to afford the pyrazole derivatives **5-8** (Scheme 3).

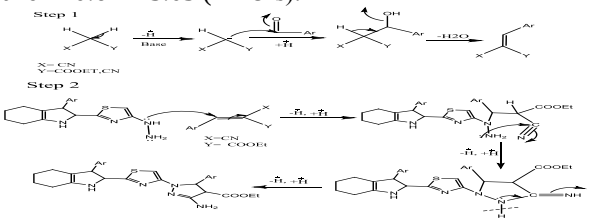


Scheme 3. Synthetic protocol of pyrazole derivatives **5-8**.



Scheme 4. Plausible mechanism for the stnthesis of compounds 5 and7 via cyclocondensation reaction condition

The structures of **5-8** have been assigned as reaction products on spectral and analytical data. In IR spectra, strong evidence for the structures is the absorption band characteristic at 1720, 1737, 1707cm^{-1} which are assigned to the ketone and ester carbonyl in case of **5**, **6** and **7**, in addition to characteristic band at 3200, 3350 cm^{-1} for NH2 group in case of **6** and **8**. The ^{1}H-NMR spectra revealed triplet signal at δ 1.22 of three protons for CH2CH3 function, one singlet of three protons for CH3 group at 2.13, quartet signal at δ 4.09 ppm owing to CH2CH3 protons, one singlet of H3 at δ 4.15ppm and one singlet of NH-pyrazole proton at δ 10.00ppm exchangeable with D2O in case of **5**, the 13C NMR spectrum of compound **5** accounted for all the carbons whose resonance appeared at the following δ values: 13.6,14.7(2CH3),21.25, 22.04, 25.84, 26.60 of cyclohexene methylene groups ;60.7,73.9(C-3),77.8(C-3"),102.3,109.1,112.3,125.4,126.6,127.3,128.3,142.0, 143.5,144.0, 151.2(C-5),164.3,166.7 (Ar-C's and hetero) which are consistent with the proposed structure for the compound. Based on the above spectral data and elemental analysis, the structure of compound **5** was confirmed as ethyl 5-methyl-3-phenyl-2-(2-(3-phenyl-1,3,4,5,6,7-hexahydro-2H-indazol-2-yl)thiazol-4-yl)-2,3-dihydro -1H-pyrazole-4-carboxylate (Scheme 3). In case of compound **6**, the NMR proton revealed a triplet signal at 1.03ppm due to CH2CH3 protons, one quartet of two protons for ester methylene at δ 4.45, it also displayed broad singlet in the range at δ 6.82 of NH2 protons, exchangeable with D2O, two doublet signal at δ 2.87 and 4.80 ppm of pyrazole-H4 and H5protons**.** Whereas, the ^{1}H-NMR spectrum of derivative **7** confirmed the presence of two singlet signal at δ1.22, 2.33 ppm due to CH3 and COCH3 protons, one singlet at δ 3.16 ppm due to pyrazole-H3and singlet at δ10.0ppm owing to pyrazole NH proton. The 13C NMR spectrum of compound **7** selected as a prototype showed the presence of the following signals at δ: 17.64(CH3), 30.03(COCH3), 56.32(C-5), 62.12(C-4), 156.70(C-3), 76.82(C-3"), 164.8(C-2'), 181.02(CO) and 110.6 -145.03 (Ar-C's).



Scheme 5. Plausible mechanism for the stnthesis of compounds **6** and **8** via cycloaddition reaction condition

On the other hand, the IR spectrum of derivative **8** displayed characteristic band at 2215cm^{-1} for C≡N group, two doublet signals at δ 2.85, 3.84 ppm due to H4 and H5 pyrazole and one singlet at δ 6.96ppm of NH2 protons with D2O exchangeable in its 1HNMR spectrum.

3.2. **Biological evaluation**

3.2.1. Antimicrobial Activity

The widespread of drug-multiresistant pathogens emphasizes the necessity for novel antimicrobial agents with lower resistance induction capacity [22]. To this end, the antimicrobial activities of the newly synthesized compounds **1-8** were evaluated against

three representative human pathogens including *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Candida albicans* using microdilution assay protocols. The results of testing compounds for antibacterial and antifungal effects are summarized in Table1. As shown in the scope of antimicrobial activity, the results depicted varied broad-spectrum antimicrobial activity for all prepared compounds. Maximum inhibition for *Streptococcus mutans* was recorded in case of compound **5** with MIC of 10.5 µg/ml showing higher antibacterial activity compared to ampicillin (MIC 13.5 µg/ml), followed by compound **8** which had moderate antibacterial activity against *Streptococcus mutans* about 58% of ampicillin. Maximum inhibition for G-ve *Pseudomonas aeruginosa* was recorded in case of compound **4** with MIC of 10.5 µg/ml, followed by compounds **3** and **8** with MIC of 20.3, 26.03 µg/ml respectively and revealed low antibacterial activity against G+ve *Streptococcus mutans* (MIC 41.5 µg/ml) compared to that of ampicillin with moderate antifungal activity against *Candida albicans* (MIC 39.9 µg/ml).

Table (1): MIC values (µg/ml) of the newly prepared compounds against three pathogenic organisms as compared to three references drugs.

Compound	Streptococcus mutans	Pseudomonas aeruginosa	Candida albicans
Ampicillin	13.5± 0.234	-	-
Ciprofloxacin	-	18.7± 0.475	-
Clotrimazole	-	-	14.5 ± 1.25
1	84.29 ± 0.5229	77.08 ± 1.884	123.7 ±1.308
2	70.26 ±0.6919	71.39 ±2.281	86.59 ±1.211
3	44.61 ±1.115	20.32 ±2.029	53.43 ±1.352
4	41.49 ±0.2597	20.2 ±1.768	39.90 ±0.189
5	10.54 ±0.4373	44.61 ±2.119	44.3 ±1.209
6	44.26 ±0.9048	43.1 ±2.065	47.81 ±1.127
7	49.14 ±0.8147	38.31 ±2.057	59.59 ±1.238
8	23.34 ±0.5382	26.03 ±1.912	50.99 ±1.122

3.2.2. Microbial-biofilm inhibition activity

Microbial-biofilm formation is one of the main pathogenicity mechanisms for microbial pathogens assisting persistence infection [23] accordingly, compounds that impair pathogens-biofilm formation increases the suitability of such microorganisms to applied drugs. In the current study, the efficacy of prepared compounds **1-8** were evaluated for microbial-biofilm inhibition using TCP technique. Results indicated that compound **3** had broad-spectrum potency against biofilm-formation with biofilm-inhibition activities of against *Streptococcus mutans* (40%), *Pseudomonas aeruginosa* (53%), and *Candida albicans* (78%). Compound **4** revealed the second effective derivative with maximum biofilm inhibition of 27, 50, 70% for the three tested organisms in order(Figure 1).

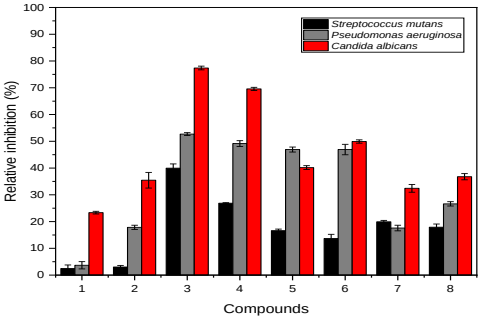


Figure 1: The effect of prepared compounds upon microbial-biofilm formation toward three tested human pathogens.

3.2.3. In vitro cytotoxic activity studies of the newly synthesized compounds

The in vitro growth inhibitory activity of the newly synthesized compounds was investigated against HFB-4, Caco-2, and hepatocellular (HepG2) carcinoma cell lines in comparison with a standard drug through colorimetric MTT assay. As usual, cytotoxic activity assaying for exploring new drugs is commonly carried out via determine cell metabolic activity using the MTT method. Hence, the MTT tetrazolium ring is cleaved to purple MTT formazan crystals by mitochondrial dehydrogenases of viable cells. Afterward, the absorbance of viable cells is proportional to their count [24,25]. In the current study, we found that the IC_{50} values of the synthesized compounds on normal cells were determined to be ranged from 40 to 168 µg/ml with higher values about 3-24 times than the IC_{50} values of cancer cells which signified its high selectivity toward cancer cells than normal cells as shown in table 2. Moreover, the results indicate that all the synthesized compounds had a potential antitumor effect against both Caco-2 cells and HepG-2 cells after treatment for 48 h with high values of SI at IC_{50} values as determined in Table 2.

Table 2: IC_{50} (µg/mL) and SI values of the synthesized compounds against both normal (HFB-4) cells and cancer (Caco-2 and HepG-2) cells after treatment for 48 h.

Cell line / Comp.	HFB-4	Caco-2		HepG-2	
	IC_{50}	IC_{50}	SI	IC_{50}	SI
1	40.72 ±2.38	12.02 ±1.04	3.39± 0.20	13.53 ±2.64	3.01± 0.18
2	65.21 ±2.99	9.69± 0.29	6.73± 0.31	6.50± 0.16	10.03 ±0.46
3	62.14 ±0.34	7.88± 0.22	7.88± 0.04	6.13± 0.13	10.14 ±0.06
4	47.41 ±3.40	14.07 ±1.99	3.37± 0.24	6.12± 0.28	7.75± 0.56
5	93.23 ±2.07	9.23± 0.11	10.10 ±0.22	8.87± 0.12	10.51 ±0.23
6	43.04 ±4.49	7.65± 0.27	5.63± 0.59	6.26± 0.57	6.88± 0.72
7	168±1 .43	7.43± 0.05	22.60 ±0.19	7.04± 0.28	23.85 ±0.20
8	48.67 ±3.64	10.33 ±0.46	4.71± 0.35	10.08 ±0.84	4.83± 0.36

All values were expressed as mean ±SD.

Among all the synthesized compounds, compound **7** showed the highest anticancer effect against both tested hepatoma and colon carcinoma with high SI values of 23.85 and 22.60, respectively, followed by compounds **5** and compound **3** with SI values of 10.51±0.23 and 10.10±0.22, respectively. In general, HepG-2 cells showed slight sensitivity toward the synthesized compounds rather than Caco-2 cells.

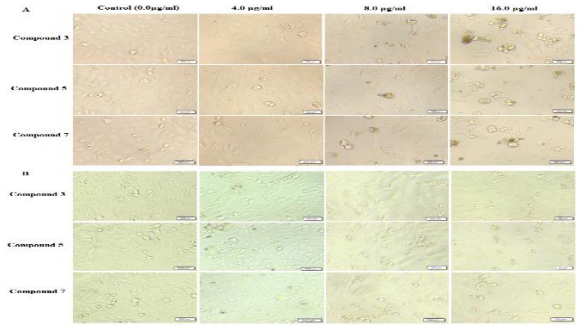


Figure 2: Effect of the synthetic compounds on the morphological modifications of cancer cells including Caco-2 (A) and HepG-2 (B) cell lines as captured under phase-contrast microscope.

Cancer cells were treated with the synthetic compounds (**3, 5,** and **7**) at different doses of 4, 8, and 16 µg/ml for 48 h, as compared to reference control cells (0.0 µg/ml).

As shown in (Figure 2) which confirms our above finding results, that revealed the relative morphological study of normal and cancer cells treated with compounds **3**, **5** and **7** at concentrations of 4, 8 and 16 µg/ml for 48 h, compared to untreated cells. All imaged micrographs were captured in a live-cell mode using phase-contrast microscopy. All captured images indicate the tested HepG-2 and Caco-2 cells were extremely affected after treatment with the tested compounds. The photomicrographs demonstrate the compounds **3**, **5**, and **7** exert a clear selectivity in cell destruction and cause cell morphological modifications in a dose-dependent manner. These observed cell modifications included blabbing, cell shrinkage, and nuclear condensation. Therefore, and based on the obtained results, it appears that the synthesized compounds have the potential to enhance apoptotic pathways to initiate their anticancer activity.

3.2.4. Effect of the potent prepared derivatives on the gene expression

Effect of the potent prepared derivatives (compounds **3, 5,** and **7**) on β-catenin, VEGF, MMP-9, P53, and Bcl-2 expression genes in both Caco-2 and HepG-2 cells lines was evaluated by qPCR technique in comparison with a standard chemotherapy drug as 5-fluorouracil (5-FU). Our results indicated that genes expression of β-catenin, VEGF, MMP-9 and Bcl-2 were suppressed in both treated Caco-2 and HepG-2 cells more than using 5-FU (Figure 3).

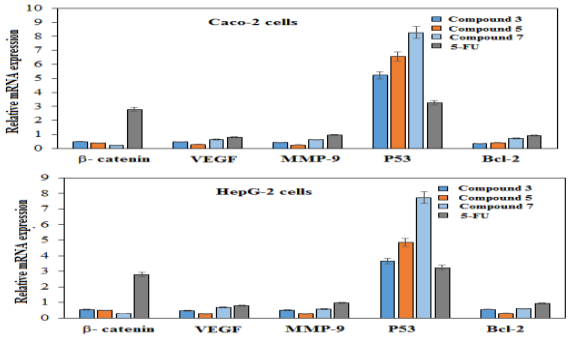


Figure 3: Assaying of the relative changes in expression levels of five key genes including β-catenin, VEGF, MMP-9, P53 and Bcl-2 using quantitative reverse transcriptase chain reaction. Angiogenesis-related genes are evaluated in Caco-2, and HepG-2 cells before and after treatment with the synthesized compounds in comparison with 5-FU for 48 h.

Furthermore, suppression in the expression level of gene Bcl-2 was significantly enhanced in the treated cancer cells by more than 2-4 folds more than untreated cells. Our results showed that the prepared compounds were successfully downregulated and inhibited expression of β-catenin gene in both cancer

treated cells in contrast to 5-FU which upregulated and active the expression of this gene. However, figure 4 shows that the expression level of p53 was upregulated clearly in treated cancer cells by more than 4-9 folds and by more than 1.3-3 folds as compared to untreated cells and 5-FU-treated cells, respectively.

3.2.5. Molecular docking studies

Molecular docking has been carried out for compounds **3, 5, and 7** toward 5 proteins namely; MMP-9, P53, β-catenin, Bcl-2, and VEGF. We aimed to study to what extent the three compounds can bind effectively to these proteins which are involved in cancer treatment. PDB ID of these proteins used in the docking study are 4XCT, 3ZME, 1JDH, 2W3L, and 2XAC, respectively. The crystal structures were downloaded and prepared for docking of our compounds and obtained data are presented in **table 3** and **table 4**.

Table 3: Binding energies of compounds **3, 5** and **7** into the examined proteins

	4XCT	3ZME	1JDH	2W3L	2XAC
The ligand	-6.53	-6.82		-5.23	
Compound 3	-5.22	-3.05	-4.94	-4.58	-3.31
Compound 5	-6.46	-5.58	-5.91	-4.71	-3.83
Compound 7	-7.03	-5.31	-5.99	-5.18	-3.65

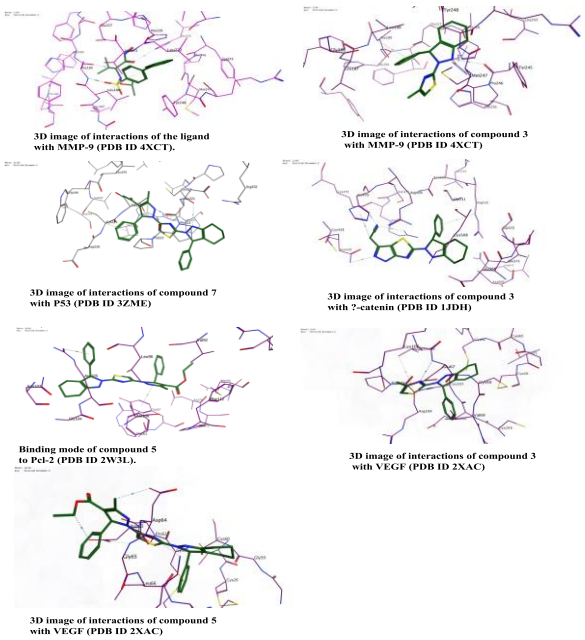
Table 4: The residues involved in the interaction of compounds 3, 5 and 7 with the selected proteins.

	4XCT	3ZME	1JDH	2W3L	2XAC
The ligand	Ala189a His236a Leu188b	Asp228a Thr 230b Cys229b		Tyr67b	
Compound 3	Tyr245a Ala189b	Glu221a Cys229b	Asn430a Lys435a Arg474a His470b Arg515b	Tyr67b	Glu67a Leu66b
Compound 5	Glu227a	Cys229b	Arg515a Arg474b	Tyr67b Arg105b	Asp63c Asp64c
Compound 7	His226b	Glu221a Cys220a Cys229b	Ser473a Arg474a Gly512a Arg612b	Tyr67b	Leu66b

[a] hydrogen bond, [b] arene-H interaction, [c] hydrophobic interaction.

The obtained data showed that the examined compounds bound less efficiently than the ligand to MMP-9 (PDB ID: 4XCT). **Figure 4** shows that 3 amino acid residues namely; Ala189, His236, and Leu188 were involved in ligand binding. We can see two hydrogen bonds with Ala189 and His236 and one arene-H interaction with Leu188. Compound **3** showed an arene-H interaction with the essential residue Ala 189 and a hydrogen bond with Tyr245 (**Fig. 5**). Notwithstanding compounds **5** and **7** exhibited relatively good binding energies, they showed binding modes differ than that of the ligand as presented in **table 3** and **table 4**.Concerning P53 (PDB ID 3ZME), it was found that the residue Cys229 is the only amino acid to which our compounds as well as the ligand bound via arene-H interaction (**Table 3**). The ligand showed a further hydrogen bond with Asp228. While compound **3** showed a hydrogen bond with Glu221. **Figure 6** shows that compound **7** showed further two hydrogen bonds with Glu221 and Cys220. The binding energies of our compounds were found to be lower than that of the ligand (**Table 3**).

Regarding β-catenin (PDB ID 1JDH), It was reported that β-catenin residues His260, Asn261, Lys292, Ile296, Asp299, Tyr306, Gly307, Lys312, Lys335, Lys345, Arg376, Arg386, Asn387, Asn426, Cys429, Lys435, Cys466, His470, Arg474 and Lys508 are the residues that interact with TCF4 to form a complex [26,27]. Hence these residues are effective ones to which the inhibitor should bind. Compound **3** showed interactions with 3 essential residues with binding energies of -4.94 Kcal/mol. Hence, it is expected to be an effective inhibitor of β-catenin. **Figure 7** shows that compound **3** formed two essential hydrogen bonds with the residues Lys435 and Arg474.



Moreover, it showed a further hydrogen bond with Asn430. It also formed arene-H interaction with the essential residue His470 and with the residue Arg515. Compound **5** exhibited arene-H interaction with the essential residue Arg474. Furthermore, it revealed a hydrogen bond with Arg515. Compound **7** showed four hydrogen bonds; one essential with Arg474 and three with Ser473, Gly512, and Arg612.

The binding pattern of the ligand to Bcl-2 (PDB ID 2W3L) showed one arene-H interaction with the amino acid Tyr67. Our compounds were able to form the same interaction with the same amino acid (Tyr67) with binding energies ranged from -4.58 to -5.18 kcal/mol. Compound 5 showed a further interaction with Arg105 (**Fig. 8).** These data suggest that our compounds are more likely to be significant inhibitors to Pcl-2.

Finally, a study of the crystal structure of VEGF (PDB ID 2XAC) suggests that the residues Asp63 and Leu66 are involved in the binding of VEGF to VEGFR via a hydrogen bond and a van der Waals interaction, respectively [28]. Accordingly, they are effective residues for the binding of inhibitors. Compound **3,** as well as compound **7,** showed an arene-H interaction with the effective residue Leu66. Compound **3** showed a further hydrogen bond with Glu67 (**Fig. 9**). Compound **5** showed two hydrophobic interactions with the essential residue Asp63 and with the residue Asp64 (**Fig. 10**). Accordingly, our compounds are expected to be potential inhibitors to VEGF.

3.2.6. Structure activity relationship(S A R)

From the results in Table 2, it is obvious that compound **2** showed higher cytotoxicity with SI values of 6.73±0.31 and10.03±0.46 than compound **1** with SI values of 3.39±0.20 and 3.01±0 due to the presence of thiazole moiety. Also, compound **3** showed higher cytotoxicity than compound **2** was probably due to the presence of the electron withdrawing group (Cl). In addition, it is clear that the pyrazole moiety was crucial for the cytotoxic effect of cyclic compounds **5-8**. Compound **7** showed the highest anticancer effect against both tested hepatoma and colon carcinoma with high SI values of 23.85 and 22.60, respectively, followed by compounds **5** with SI values of 10.51±0.23. The presence of the electron withdrawing ethanone group (COCH3) in **7** was probably responsible for its higher activity compared to compound **5**. In conclusion, based on the presented data, the presence of thiazole, pyrazole moieties and an electron withdrawing groups enhanced the potency of the compounds compared to the starting indazole **1**.

2.1.1. Structure activity relationship(S A R)

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CONCLUSIONS

In conclusion, the present study reports the facile synthesis of a potent and selective series of pyrazole derivatives (**5-8**) via indazole-2-carbothioamide (**1**), Indazolylthiazol-4-one derivative (**2**), 4-chloro indazolylthiazole(**3**) and 4-hydrazineylindazolyl thiazole (**4**) using simple reagents. Most preparations revealed broad-spectrum antimicrobial activity with potent antibacterial activity through compound **5** against *Streptococcus mutans* exceeding that of applied reference antibiotics ampicillin. In the case of G-ve bacteria *Pseudomonas aeruginosa*, compound **4** and **3** exerted the highest antibacterial activity about 92% of reference Ciprofloxacin activity. On the other hand, compounds understudy revealed moderate to low antifungal activity against *Candida albicans*. All the synthesized derivatives were screened for their in vitro anti-cancer activities against Caco-2 and hepatocellular (HepG2) carcinoma cell lines. The

results of the cytotoxic studies of the tested compounds revealed that the synthesized compounds had a potential antitumor effect against both Caco-2 cells and HepG-2 cells. Compound **7** exhibited a significant inhibitory effect on both tested hepatoma and colon carcinoma cell lines amongst all the compounds synthesized, whereas compounds **5** and **3** exhibited good cytotoxic activity. On the other hand, we found that the IC50 values of the synthesized compounds on normal cells were determined to be ranged from 40 to 168 μg/ml with higher values about 3-24 times than the IC50 values of cancer cells which signified its high selectivity toward cancer cells than normal cells. Furthermore, the molecular docking study explored a significant activity of these compounds to inhibit the interaction between β-catenin and TCF-4 (T cell Factor 4) which enhances the apoptosis process.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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