Original Article

Synthesis and Characterization of Some Novel N-Phenylpyrazole Analogues for the Evaluation of their Potentials as Anticancer, Antimicrobial and Antioxidant Agents

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ABSTRACT

Objectives: With a view to invent new anticancer agents, the authors proposed to prepare a series of N-phenylpyrazole derivatives by introducing biologically active pharmacophores viz., Fluoro/Fluoromehtyl benzamide and 2, 3-dihydrobenzo[b][1,4] dioxin at 3-, 5- positions of N-phenyl pyrazole motif respectively. **Methods:** The newly formed products are characterized by ^{1}H NMR, ^{13}C NMR, Mass and FTIR spectroscopic techniques and are subjected to screened for anticancer activity against human liver cancer cell line (Hep G2), antimicrobial and antioxidant activities. Further, Molecular docking study has also been applied on the newly synthesized compounds to study the binding efficiencies with protein BCL2 using GOLD docking software. **Results:** Among all the newly synthesized compounds, three compounds 8(d), 8(e), 8(h) exhibited higher potentials of anticancer activity compared to the rest of the compounds. All the newly synthesized compounds exhibited antimicrobial and antioxidant activities. Further study of molecular docking with protein BCL2 revealed that three title compounds 7, 8(f) and 8(h) exhibited very good binding efficiencies.

Key words: Anticancer, BCL2, Chalcone, Hep G2, Molecular docking, Pyrazole.

INTRODUCTION

In the present scenario, Cancer becoming a biggest threat at global level and is the second most leading cause for the deaths. Nearly 70% of mortality from cancer occurs in low income of nations. About 33.33% of cancer mortalities are due to the five major behavioural and dietary risks: i). Obesity ii). Less fruit consumption iii). Lack of physical exercise iv). Usage of tobacco and consumption of alcohol.[1]

Since last two decades, heterocyclic rings bearing pyrazole, especially N-Phenyl pyrazoles, attracts the scientists to invent a new type of anticancer agents in the area of drug discovery, medicinal and pharmaceutical chemistry. Recent literature also revealed that N-phenylpyrazoles were found with very good biological activities viz., antifungal, anti-bacterial and anticancer activities.[2] In addition, some of the pyrazole derivatives are also exhibiting versatile inhibitory activities including, c-Jun N-terminal kinase,[3] CGT1 inhibitors,[4] BRAFV^{600E4} inhibitors,[5] CDKs inhibitors,[6] BACE1 inhibitors,[7] telomerase inhibitors,[8] xanthine oxidoreductase inhibitors,[9] COX-2 inhibitors[10] etc., Besides the synthetic pyrazoles, natural products bearing pyrazole motif are also of great value in our daily life.[11]

Keeping in view, the recent research work and the innovations in the discovery of

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new anticancer, antibacterial, antifungal and antioxidant agents, the authors focused on the synthesis and characterization of a series of novel analogues of N-phenylpyrazole linked with biologically active pharmacophores viz., Fluoro/Fluoromehtyl benzamide and 2, 3-dihydrobenzo[b] [1,4]dioxin and assessing their anticancer activities against human liver cancer cell line (HepG2). Further study on molecular docking has also been carried out with protein BCL2 to assess the binding efficiencies of the newly synthesized pyrazole derivatives since N-phenyl pyrazole derivatives are acting as BCL2 inhibitors.[12]

MATERIALS AND METHODS

All the materials employed in the present research work are of LR grade (AVRA Synthesis Pvt Ltd, Hyderabad) and of commercial grade with purity more than 95% and were used as such. Melting ranges of all the synthesized compounds were determined using POLMON (MP96) instrument. Recorded the FTIR spectra on Shimadzu instrument (IRAffinity-1S) applying Attenuated Total Reflection technology. Recorded the ^{1}H NMR, ^{13}C NMR spectra on BRUCKER (400 MHz) in DMSO-d_6 using trimehtylsilane as internal reference. The chemical shift values, coupling constants (*J*) are reported in ppm, Hz respectively. Recorded mass spectra on Agilent 6120 spectrometer using ESI-API ionisation technique. Progresses of the reactions were monitored by using Merck silica gel 60F_{254} (105554) plate eluting with a suitable mobile phase and were visualized under a UV light at a wave length of 254 nm. Purified all the newly synthesized compounds by column chromatography packed with Silica (SiO_2, 60-120 mesh) eluting a suitable gradient mobile phase.

Anticancer Evaluation

Human liver cancer cell lines (Hep G2) were purchased from national centre for cell science (Pune) and stored in a growth media (RPMI 1640) and 10% Fetal bovine serum (#RM10432, Himedia) was provided as supplement. The cells were kept for confluency in an incubator by maintaining feasible conditions; temperature 37°C with humidified atmosphere of 5% carbon dioxide. There was seeded 200µl cell suspension in a well plate (96 well type model) at minimum mandatory density (20,000 cells/well) without adding the test reagent. After that the cells were left for develop over a period of 12 hr. The test samples of various concentrations 25, 50, 75, 100 and 125 µg/ml of synthesized compounds and 50 µg/ml of reference drugs were added for the growth of the cells separately. The plates were placed for incubation for a period of 24 hr at temperature about 37°C and 5% carbon dioxide atmosphere. After incubation time, the plates were taken out and then discarded the spent media. Now the plates were again kept on incubation for 3 hr, by adding MTT reagent to the final concentration of 0.5 mg/ml of total volume. After removal of MTT reagent, 100 µl of DMSO was added. The absorbance was recorded on an ELISA reader at a wave lengths 570 and 630 nm. The IC_{50} values were calculated by drawing a linear relation i.e., y=mx+c. The values m and c were calculated from the viability graph by considering y=50. The IC_{50} value is also calculated by taking "Sorafenib" as reference standard drug substance.

Antimicrobial Activity

Salmonella enterica (MTCC 1253), *Pseudomonas aureoginosa* (MTCC 2453), *Bacillus cereus* (MTCC 1305), *Streptococcus pyrogenes* (MTCC 442) and *Aspergillus niger* were selected based on their pharmacological and clinical importance. The microbial strains received from Microbiology Department, Sri Yuva Biotech Pvt. Ltd., and were used for the evaluation of antimicrobial activity. The cultures were placed in an incubator at 37°C for a period of 24 hr on nutrient agar. Microbial growth was observed on Mueller-Hinton agar plates at a 37°C. Simultaneously, the stock cultures were also maintained at 4°C. For the growth of fungi, potato dextrose agar was used.

Determination of zone of inhibition method

Whatman (No.1) filter paper discs of diameter 5mm were autoclaved initially by keeping in a clean and dry petri plate. The discs were placed in the organic compound solutions for soaking for 5 hr. After soaking, the discs were slowly shade dried and are used for test material. The concentration of compound solutions maintained at 200, 300 and 500 µg/mL. Subsequently, transferred the resultant solutions of various concentrations to spread on cultured petri plates. Filter paper discs dipped in reference drug "streptomycin" of various concentrations 200, 300 and 500 µg/mL and were used for reference study.

Testing of antibacterial and Antifungal activity

The antimicrobial activity was measured in LB agar medium and was sterilized by keeping at 121°C for a period of 30 min. Agar paltes were arranged by applying this agar medium (about 10 mL) in petri dishes (10 cm) and left for 2 hrs under aseptic environment without disturbing to make the medium solid. Now, 1 mL of inocculum of microbial species (i.e., *Salmonella enterica/Pseudomonas aureoginosa/Bacillus cereus/Streptococcus Pyrogenes/ Aspergillus niger)* was placed separately on the plates presented with solid agar media. The previously prepared sterile paper discs were saturated with the

compound solutions and then incubated for a period of 48 hrs in BOD at 37°C for the growth of inhibitory zones. After incubation period, the mean values of the three independent readings for each micro-organism at various concentration levels of the synthesized compounds were recorded. The inhibition zone diameter was measured and then recorded with a plastic ruler by keeping 5 to 6 discs in one petri plate.

Antioxidant Activity

The antioxidant activities of all the isolated compounds have been evaluated by adopting DPPH and ABTS protocol methodologies. In this context, reference and test samples have been prepared of concentration 100μg/mL in methanol as diluent.

Antioxidant Evaluation-DPPH Assay methodology

The DPPH radical cation protocol has been modified for the evaluation of radical scavenging effect of all the isolated twelve compounds. Dissolved DPPH reagent (8 mg) in Methanol (100 mL) for the preparation required concentration of 80 *μ*L/mL. For determining scavenging activity, mixed DPPH (100 μL) reagent with test sample (100μL) in a 96 well micro plate and then kept for incubation for a period of 30 min. After completion of the incubation period, recorded the absorbance readings at 514 nm by using ELISA reader by using methanol (100%) as control and adjusting the concentration level to 100μg/mL. The radical scavenging activity was calculated by substituting the values in the standard formula;

Radical scavenging (%)=$[(A_{control}-A_{sample}) / A_{control}]\times 100$.
Finally, The IC_{50} values were calculated by adopting the methodology of regression analysis for evaluation.

Antioxidant Evaluation-ABTS Assay methodology

The ABTS radical scavenging methodology has been modified for the evaluation of free radical-scavenging effect of twelve newly isolated compounds. For the preparation of ABTS reagent, mixed 5 mL of ABTS (7mM) reagent with 88μL of $K_2S_2O_8$ (140 mM). The resultant mixture was placed in dark room at 25-30°C for 16 hr, to generate free radicals. After a period of 16 hrs, the mixture was again diluted with water in 1:40 ratio. ABTS reagent (100 μL) was mixed with sample (100 μL) in a 96 well plate and then placed in an incubator at 25-30°C for 6 to 7 min. After completion of the incubation time, absorbance values were recorded at 734 nm. Finally radical scavenging activities, IC_{50} values were calculated as mentioned in the DPPH methodology for the evaluation of antioxidant activities.

Molecular Docking Study

Study of docking analysis carried out to assess the binding efficiencies with protein BCL2 (B-cell lymphoma 2).

Methodology

Structure of BCL2 of Homo sapiens (Protein data base ID: 5JSN) was generated from protein data base.[13] After removal of the unwanted hetero atoms and chains using surface and electrostatic properties of proteins (SPDBV) software and then added hydrogen's to the protein for the identification of active site. Finally, the obtained stable structure of BCL2 is as shown in Figure 1. Besides the hetero atoms; the ligands present in the crystal structure are also removed for docking analysis.

Docking method

Molecular docking has been done using GOLD software which is based on Genetic Algorithm (GA). This algorithm allows flexibility of protein partially and flexibility of ligands fully. The newly prepared compounds are subjected to dock at the active site of BCL2. The interaction between the test compounds with the active site residues are investigated by applying molecular mechanics theory. The main parameters generally using in genetic algorithm (GA) include; i).population size (100) ii).selection pressure (1.1) iii). number of operations (10,000) iv).number of island (1) and niche size (2). The operator parameters like., crossover, mutation and migration were adjusted to 100, 100 and 10 respectively. Applied default cut off values for hydrogen's and vanderwaals as 3.0 A° (dH-X) and

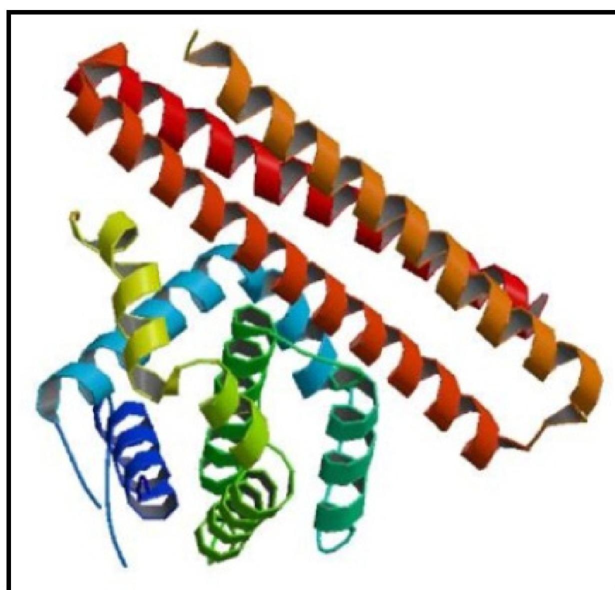


Figure 1: Structure of BCL2.

6.0 A° respectively. During the analysis of molecular docking, the speed of default algorithm and the binding site of ligand in the BCL2 were defined within a specific radius of 10 A° with the centroid as CE atom of ASP 25. Number of poses for every inhibitor was adjusted to 100 and allowed to the early termination if the first three binding conformations of the ligand were within 1.5A° RMSD. After completion of docking process, the interactions and binding poses of each ligand were studied. Finally selected more stable and favourable confirmation for each individual ligand (Verdonk *et al.* 2005).[14]

Gold Score fitness function

The GOLD score function consists four main elements and are

i). Protein-ligand vander Waals energy (external vdw); ii). Ligand intramolecular hydrogen bond energy (internal- H- bond); iii). Protein-ligand hydrogen bond energy (external H-bond); iv). Ligand internal vander Waals energy (internal vdw). Total fitness score was calculated by the multiplication of external vdw score with factor of 1.375 which is an empirical correction to support protein-ligand hydrophobic contact. Finally, the optimised fitness function to predict the binding positions of ligands is;

Gold Score =S(hb_ext)+S(vdw_ext)+S(hb_int)+S (vdw_int)

here, S(hb_ext) denotes the hydrogen bond score of protein-ligand; S(vdw_ext) denotes vanderwaals score of the protein-ligand, S(hb_int) denoted the resultant score from intramolecular hydrogen bond in the ligand; S(vdw_int) denotes the resultant score from intramolecular strain in the ligand.

Active site Identification of BCL2

Active site of BCL2 protein was recognized using computed atlas of surface topography of proteins (CASTp) server which is based on defined computational geometrical methods, include alpha shape and discrete flow theory. The main feature of the CASTp programme is that to locate and measure the protein pockets and cavities automatically and also measures the pockets and pocket mouth openings and cavities. This programme specified for lining pockets of the atoms, buried cavities and pocket openings; the area of pockets, volumes and cavities, circumference and area of mouth openings (Liang *et al.* 1998).[15]

Once the model finalised, searched the probable binding sites of BCL2 by considering structural comparison of template as basis. Finally the resultant active site of BCL2 protein is as shown in Figure 2. Further, it was also observed that 2° structures are highly preserved and the residues, ASP25, LEU23, ILE84, ARG87, SER133, ASP132, VAL 134, PHE136, ASP135, GLN152, THR243, GLU242, THR244, ASN144.

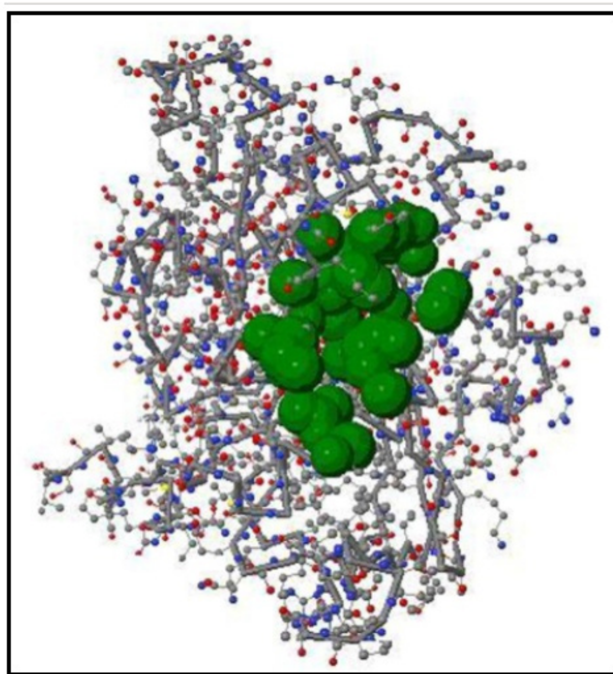


Figure 2: Active site of BCL2.

Synthesis

2, 3-Dihydrobenzo[b][1, 4] dioxine-6-carbaldehyde (2)[16]

To a stirred suspension of 3,4-dihydroxybenzaldehyde 1 (50 g, 362 mmol) and caesium carbonate (236 g, 724 mmol) in DMF (500 ml, 1.38 ml/mmol), added 1,2-dibromoethane (136 g, 724 mmol) slowly by drop wise at 25 to 35°C. Heated the contents to 75-85°C for 8 hr. Reaction was monitored by using TLC (30 % v/v, EtOAc in petroleum ether, R_f: 0.74). Once reaction is completed, Reaction mass was cooled and then poured in water (1000 ml). Extracted the compound with EtOAc (3X150 ml).Washed the total EtOAc layer with 200 mL of water and dried with anhydrous Na_2SO_4. Evaporated Ethyl acetate under vacuum and then purified the obtained crude by column chromatography eluting with petroleum ether to 5 % v/v EtOAc in petroleum ether furnished the title compound 2 (42.5 g, 71.5 %) as white solid. M.P: 52-55°C. IR spectrum, ν, cm^{-1}: 1682 (C=O), ^{1}H NMR(400 MHz, DMSO-d_6): 4.354-4.302 (dd, 4H, J_1=4.4 Hz, J_2=17.2 Hz), 7.060-7.040 (d, 1H, Ar-H, J=8 Hz), 7.437-7.372 (t, 2H), 9.806 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): 64.36, 65.06, 118.14, 118.26, 124.14,

130.72, 144.20, 149.50, 191.67 Mass: Calculated: 164.16 found: 165.2 (100%, $[M+H]^+$), 166.2 (12 %, $[M+2H]^+$).

4-((E)-3-(2,3-Dihydrobenzo[b][1,4]dioxin-7-yl) acryloyl]benzonitrile (4)[17]

To a stirred solution of 2,-3-Dihydrobenzo[b][1, 4] dioxine-6-carbaldehyde 2 (25 g, 152.3 mmol) and 4-acetylbenzonitrile 3 (22.1 g, 152.3 mmol) in methanol (500 ml, 3.28 ml/mmol), aqueous solution of 2N NaOH (84 ml, 167.5 mmol) was added slowly by drop wise at 10-15°C over a period of 20-30 min. Allowed the reaction mass to 25-35°C for 8 hr. Monitored the reaction with TLC (30 % v/v, EtOAc in petroleum ether, R_f: 0.67). added water (100 ml) and then stirred for 1 hr. Filtered the obtained yellow coloured solid, Drying of the solid at 55-65°C furnished the title compound 4 (35 g, 79%) as yellow solid. M.P: 173-175°C. FTIR ($cm^{-1)}$:2229.71 (-C≡N), 1653.00 (-C=O). ^1H NMR(400 MHz, DMSO-d_6): 4.325-4.288(m, 4H), 6.955-6.934 (d, 1H, J=8.4 Hz), 7.410-7.384 (dd, 1H), 7.539-7.534 (d, 1H, 7.720-7.681 (d, 1H, J=15.6), 7.836-7.797 (d, 1H,J=15.6 Hz), 8.059-8.039 (d, 2H, J=8), 8.298-8.278 (d, 2H, J=8 Hz)^{13}C NMR (400 MHz, DMSO-d_6): 64.42, 64.92, 115.34, 117.89, 117.95, 118.74, 120.15, 123.94, 128.42, 129.51, 133.23, 141.53, 144.11, 145.78, 146.71, 188.66 Mass: Calculated

291.3 found: 292.2(100%, $[M+H]^+$), 293.2 (21%, $[M+2H]^+$).

4-(5-(2,3-Dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl)benzonitrile (5)[18]

To a stirred suspension of compound 4 (4.0 g, 13.73 mmol) and phenylhydrazine (5.90 g, 54.55mmol) in Ethanol (150 ml, 10.9 ml/mmol), acetic acid (82 mg, 1.36 mmol) was added at 25-35°C. To this reaction mixture, molecular iodine (6.9 g, 27.18 mmol) was added at 25-35°C slowly over a period of 10-15 min (slightly exothermic reaction). Heated the reactants for 8 hr at reflux and cooled the reaction mixture to 0-5°C. Filtered the obtained brown coloured solid and then washed with hexane. Drying of the material in a hot air oven at 45-55°C furnished title compound 5 (4.7 g, 90%) as brown coloured solid. M.P: 165-167°C, Chemical formula

$C_{24}H_{17}N_3O_2$ FTIR (cm^{-1}): 2225.85 (-C≡N), 1610.56 (-C=O). ^1H NMR(400 MHz, DMSO-d_6):4.25-4.24 (d, 2H, *J*=4.0), 6.70-6.72 (d, 1H, *J*=8.4), 6.81-6.86 (t, 2H), 7.27 (s, 1H), 7.37-7.39 (d, 2H), 7.44-7.50 (m, 3H), 7.91-7.93 (d, 2H, *J*=8.0), 8.09-8.11 (d, 2H, *J*=8.0).^{13}C NMR (400 MHz, DMSO-d_6): 64.50, 64.63, 106.21, 110.68, 117.60, 117.75, 119.39, 122.04, 123.03, 125.90, 126.40, 128.64, 129.65, 133.30, 137.64, 140.13, 143.71, 144.28, 144.69, 149.60 Mass: Calculated: 379.13 found: 380.0 (100%, $[M+H]^+$), 381.2 (28%, $[M+2H]^+$), 382.2 (14%, $[M+3H]^+$).

Ethyl-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzoate (6)

Compound 5 (4 g, 10.55 mmol) was added to Ethanol-HCl (30 ml, 24% w/v) in a 50 ml pressure tube at 25-35°C and then heated the reaction mixture to reflux for 3 hr by protecting the entire reaction using a safety shield. Reaction was cooled to 5°C and then released HCl gas very slowly. Poured the reaction contents in cold water (100 ml). Neutralized the reaction mass with solid sodium bicarbonate slowly. Compound was exctracted with EtOAc (3X25 ml) and then washed the EtOAc layer with water (2X25 ml), brine solution (1X25 ml). Finally dried the EtOAc layer over anhydrous Na_2SO_4. Evaporated EtOAc and then purification of the obtained crude by column chromatography eluting with petroleum ether to 50% EtOAc in petroleum ether furnished the title compound 6 (2.76 g, 61%) as white solid. M.P:168-170°C, FTIR (cm^{-1}):1697.36 ((-C=O). ^1H NMR(400 MHz, DMSO-d_6): 1.37-1.33 (t, 3H), 4.25-4.24 (d, 4H), 4.37-4.32 (q, 2H), 6.73-6.71 (d, 1H, *J*=8.4), 6.86-6.81 (t, 2H), 7.22 (s, 1H), 7.39-7.37 (d, 2H, *J*=7.6), 7.50-7.41 (t, 3H), 8.07-8.02 (t, 4H). ^{13}C NMR (400 MHz, DMSO-d_6): 14.66, 61.21, 64.50, 64.63, 105.97, 117.56, 117.73, 122.03, 123.14, 125.86, 125.90, 128.53, 129.51, 129.62, 130.16, 137.62, 140.21, 143.70, 144.22, 144.53, 150.16, 166.01. Mass: Calculated: 426.46 for $C_{26}H_{22}N_2O_4$ found: 427.2 (100%, $[M+H]^+$), 428.0 (25%, $[M+2H]^+$).

4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl)benzoic acid (7)

To a stirred solution of Ethyl-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl)benzoate 6 (2.6 g, 6.09 mmol) in THF (26 ml, 4.27ml/mmol), a solution of Lithium hydroxide monohydrate (0.76 g, 18.11mmol) in water (9 ml) was added. The resultant reaction mixture was heated to reflux for 6 hr. Checked the progress by TLC (EtOAc: petroleum ether, 1:1; R_f: 0.38). After reaction, evaporated THF completely under reduced pressure. Added water (50 ml) to the mass and then washed with EtOAc (2X25 ml). Acidified the aqueous layer with aq 2N HCl solution. Extracted the compound using EtOAc (3 X 30 ml). Washed the total EtOAc layer with brine solution (50 ml) and then finally dried with Na_2SO_4. Evaporated EtOAc under vacuum completely. Washed the obtained solid with EtOAc (10 ml) furnished the title compound 7 (1.90 g, 78 %) as white solid.M.P:224-226°C, FTIR (cm^{-1}):1674.21 (-C=O). ^1H NMR(400 MHz, DMSO-d_6): 4.06-4.00 (q, 4H), 6.72-6.70 (d, 1H, *J*=8), 6.85-6.80 (t, 2H), 7.19 (s,

1H), 7.38-7.37 (d, 2H, J=7.2), 7.48-7.42 (m, 3H), 8.02 (m, 4H), 12.95 (bs, 1H)^{13}C NMR (400 MHz, DMSO-d_6): 64.50, 64.62, 105.94, 117.57, 117.73, 122.03, 123.19, 125.75, 125.86, 128.47, 129.60, 130.34, 130.45, 137.29, 140.23, 143.70, 144.21, 144.48, 150.30, 167.58Mass: Calculated: 398.41for $C_{24}H_{18}N_2O_4$ found: 399.2 (100%, $[M+H]^+$), 400.0 (23%, $[M+2H]^+$).

General procedure for the synthesis of title compounds 8(a-h)

Oxalylchloride (3.0 mmol) was added to a stirred solution of compound 7 (1.0 mmol) in DCM (15 ml) and DMF (0.05 ml) at 0-5°C slowly and then stirred for 2 hr at 0-5°C. After 2 hr, evaporated solvents and oxalyl chloride completely under reduced pressure. Dissolved the obtained crude in DCM (15 ml) and then added the resultant acid chloride in DCM to a stirred solution of corresponding aniline 7a-h (1.0 mmol), DIPEA (3.0 mmol) in DCM (15 ml) at 0-5°C. Allowed the reaction mass to 25-35°C for 3 hr. Reaction was monitored with TLC. After reaction, DCM (15 ml) was added to the reaction mass. Washed the resultant DCM layer with water (25 ml), aq HCl (25 ml) and saturated $NaHCO_3$ solution (25 ml) respectively. Finally washed the organic layer with brine (25 ml) and then dried with anhydrous Na_2SO_4. Distilled DCM completely by applying vacuum. Purification of the obtained crude by column chromatography eluting with petroleum ether to 50% DCM in petroleum ether furnished the title compounds 8a-h.

N-(2-Fluorophenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide8(a)

67%, white solid, M.P: 184-186°C. FTIR (cm^{-1}): 1651.07 (-C=O), 1614.42 (-NH)^{1}H NMR (400 MHz, DMSO-d_6): 4.26-4.23 (q, 4H), 6.73-6.71 (dd, 1H), 6.86-6.82 (dd, 2H), 7.25-7.21 (m, 2H), 7.34-7.27 (m, 2H), 7.40-7.38 (m, 2H), 7.50-7.42 (m, 3H), 7.63-7.59 (q, 1H), 8.07 (s, 4H), 10.18 (s, 1H) ^{13}C NMR (400 MHz, DMSO-d_6):64.01, 64.13, 105.44, 115.72, 115.92, 117.08, 117.25, 121.53, 122.73, 124.30, 125.13, 125.42, 125.67, 127.22, 128.00, 128.36, 129.13, 133.00, 135.90, 139.80, 143.21, 143.71, 143.96, 149.90, 164.98Mass: Calculated:491.16for $C_{30}H_{22}FN_3O_3$found: 492.2 (100%, $[M+H]^+$), 493.2 (30%, $[M+2H]^+$), 490.2 (100%, $[M-H]^+$), 491.0 (30%, $[M]^+$).

N-(3-Fluorophenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide8(b)

64%, white solid, M.P: 174-176°C. FTIR (cm^{-1}):1651.07 (-C=O), 1595.13 (-NH), ^{1}H NMR (400 MHz, DMSO-d_6): 4.26-4.23 (q, 4H), 6.73-6.71 (dd, 1H), 6.86-6.82 (dd, 2H), 6.97-6.92 (m, 1H), 7.24 (s, 1H), 7.50-7.38 (m, 6H), 7.60-7.58 (d, 1H), 7.80-7.76 (m, 1H), 8.08-8.03 (q, 4H),10.48 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6):64.51, 64.63, 105.95, 107.35, 107.61, 110.45, 110.66, 116.47, 116.50, 117.58, 117.74, 122.03, 123.23, 125.63, 125.90, 128.47, 128.81, 129.62, 130.64, 130.74, 134.18, 136.39, 140.27, 141.42, 141.52, 143.71, 144.21, 144.47, 150.36, 161.36, 163.75, 165.89 Mass: Calculated: 491.16 for $C_{30}H_{22}FN_3O_3$ found: 492.2 (100%, $[M+H]^+$), 493.2 (30%, $[M+2H]^+$), 494.2 (8%, $[M+H]^+$).

N-(4-Fluorophenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide8(c)

70%, white solid, M.P: 255-257°C. FTIR (cm^{-1}): 1635.64 (-C=O), 1612.49 (-NH), ^{1}H NMR (400 MHz, DMSO-d_6): 4.26-4.23 (q, 4H), 6.73-6.71 (dd, 1H), 6.86-6.82 (dd, 2H), 7.23-7.18 (t, 3H), 7.49-7.38 (m, 5H), 7.84-7.80 (q, 2H), 8.05 (s, 4H),10.35 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6):63.97, 64.10, 105.38, 115.00, 115.22, 117.03, 117.20, 121.48, 122.12, 122.20, 122.70, 125.05, 125.37, 127.93, 128.17, 129.03, 133.82, 135.47, 135.66, 139.74, 143.17, 143.67, 143.91, 149.85, 157.03, 164.97 Mass: Calculated: 491.16 for $C_{30}H_{22}FN_3O_3$ found: 492.2 (100%, $[M+H]^+$), 493.0 (26%, $[M+2H]^+$).

N-(2-(Trifluoromethyl)phenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide 8(d)

62%, white solid, M.P: 166-168°C. FTIR (cm^{-1}):1689.64 (-C=O), 1610.56(-NH)^{1}H NMR (400 MHz, DMSO-d_6): 4.26-2.51 (q, 4H), 6.74-6.72 (dd, 1H), 6.87-6.82 (q, 2H), 7.22 (s, 1H), 7.40-7.38 (t, 2H), 7.50-7.43 (q, 3H), 7.58-7.54 (t, 2H), 7.78-7.74 (t, 1H), 7.83-7.81 (d, 1H, J=7.6), 8.08-8.03 (q, 4H), 10.20 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6): 64.51, 64.63, 79.09, 79.42, 79.75, 105.92, 117.57, 117.74, 122.04, 123.24, 125.68, 125.90, 126.94, 128.47, 128.70, 129.62, 131.76, 133.51, 133.59, 136.40, 140.26, 143.70, 144.20, 144.46, 150.37, 166.46 Mass: Calculated: 541.16 for $C_{31}H_{22}F_3N_3O_3$ found: 542.2 (100%, $[M+H]^+$), 543.2 (36%, $[M+2H]^+$), 544.2 (7%, $[M+3H]^+$).

N-(3-(Trifluoromethyl)phenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide 8(e)

66 %, white solid, M.P: 167-169°C. FTIR (cm^{-1}): 1645.28 (-C=O), 1614.42 (-NH) ^{1}H NMR (400 MHz, DMSO-d_6): 4.25-4.24 (d, 4H, H=3.6), 6.74-6.71 (dd, 1H), 6.86-6.82 (q, 2H), 7.24 (s, 1H), 7.40-7.38 (t, 2H), 7.50-7.43 (m, 4H), 7.63-7.60 (t, 1H), 8.08 (s, 5H), 8.28 (s, 1H), 10.59 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6): 64.51, 64.63,

105.95, 116.85, 116.90, 117.57, 117.74, 120.42, 122.03, 123.22, 123.30, 124.29, 125.65, 125.90, 126.00, 128.49, 128.82, 129.62, 129.69, 130.00, 130.34, 133.96, 136.49, 140.26, 140.47, 143.71, 144.21,144.48, 150.34, 165.99 Mass: Calculated: 541.16 for $C_{31}H_{22}F_3N_3O_3$ found: 542.2 (100%, $[M+H]^+$), 543.2 (36%, $[M+2H]^+$).

N-(4-(Trifluoromethyl)phenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide 8(f)

62 %, white solid, M.P: 251-253°C. FTIR (cm^{-1}): 1647.21 (-C=O), 1610.56 (-NH) ^{1}H NMR (400 MHz, DMSO-d_6): 4.25-4.24 (d, 4H, J=4), 6.74-6.72 (t, 1H), 6.86-6.82 (q, 2H), 7.23 (s, 1H), 7.40-7.38 (d, 2H), 7.50-7.43 (m, 3H), 7.75-7.73 (d, 2H), 8.08-8.04 (t, 6H), 10.62 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6): 64.51, 64.63, 105.95, 117.57, 117.73, 120.63, 122.03, 123.21, 123.53, 123.92, 124.24, 124.56, 125.63, 125.90, 126.23, 126.36, 126.40, 128.49, 128.90, 129.62, 134.02, 136.51, 140.26, 143.32, 143.70, 144.21, 144.48, 150.33, 166.10 Mass: Calculated: 541.16 for $C_{31}H_{22}F_3N_3O_3$ found: 542.2 (100%, $[M+H]^+$), 543.2 (36%, $[M+2H]^+$), 544.2 (6%, $[M+3H]^+$), 540.0 (100%, $(M-H]^+$), 541.2 (23%, $[M]^+$), 542.2 (5%, $[M]^+$).

N-(2,6-Difluorophenyl-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide 8(g)

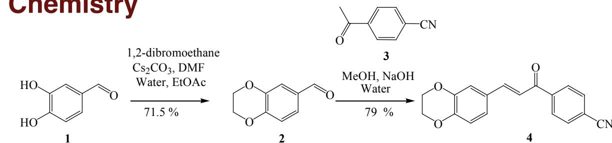
68 %, white solid, M.P: 203-204°C. FTIR (cm^{-1}): 1658.78 (-C=O), 1614.42 (-NH), ^{1}H NMR (400 MHz, DMSO-d_6): 4.26-4.23 (q, 4H), 6.74-6.71 (dd, 1H), 6.86-6.82 (q, 2H), 7.25-7.21 (q, 3H), 7.50-7.38 (m, 6H), 8.08 (s, 4H), 10.18 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6): 64.00, 64.12, 105.45, 111.76, 111.99, 117.07, 117.23, 121.52, 122.72, 125.22, 125.41, 127.98, 128.36, 129.11, 132.17, 139.76, 143.20, 143.70, 143.98, 149.79, 165.00 Mass: Calculated: 509.16 for $C_{30}H_{21}F_2N_3O_3$ found: 510.2 (100%, $[M+H]^+$), 511.2 (37%, $[M+2H]^+$), 512.2 (7%, $[M+3H]^+$).

N-(4-Bromo-3-(trifluoromethyl)phenyl)-4-(5-(2,3-dihydrobenzo[b][1,4]dioxin-7-yl)-1-phenyl-1H-pyrazol-3-yl) benzamide 8(h)

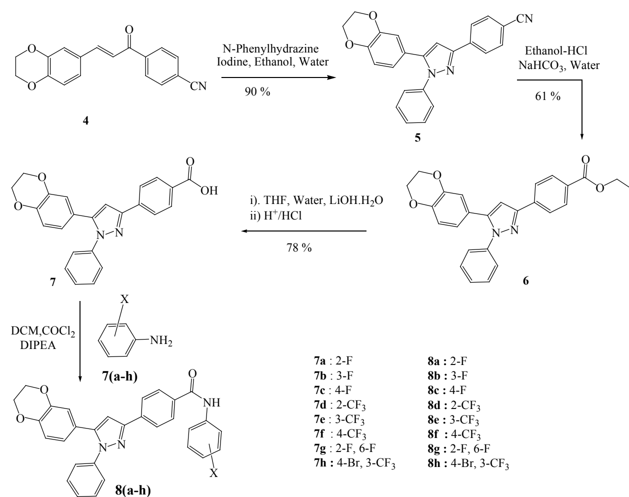
61 %, white solid, M.P: 99-102°C. FTIR (cm^{-1}): 1651.07 (-C=O), 1614.42 (-NH) ^{1}H NMR (400 MHz, DMSO-d_6): 4.26-4.25 (d, 4H, J=2.4), 6.74-6.72 (d, 1H, J=7.6), 6.87-6.83 (t, 2H), 7.25 (s, 1H), 7.41-7.39 (t, 2H), 7.49-7.44 (m, 3H), 7.90-7.88 (d, 1H, J=8.4), 8.09 (s, 5H), 8.34 (s, 1H), 10.68 (bs, 1H) ^{13}C NMR (400 MHz, DMSO-d_6): 64.51, 64.64, 105.98, 112.33, 117.58, 117.74, 119.78, 122.03, 123.21, 124.74, 125.45, 125.67, 125.90, 128.49, 128.86, 129.08, 129.62, 133.70, 135.85, 136.63, 139.67, 140.26, 143.71, 144.21, 144.48, 150.31, 166.60 Mass: Calculated: 619.07 for $C_{31}H_{21}BrF_3N_3O_3$ found: 620.0 (100%, $[M+H]^+$), 622.0 (85 %, $[M+3H]^+$), 623.0 (25%, $[M+4H]^+$), 624.0 (5%, $[M+5H]^+$).

RESULTS AND DISCUSSION

Chemistry



Scheme-1 : Synthetic pathway for the synthesis of title Compound-4



Scheme-2 : Synthetic pathway for the synthesis of title compounds 8(a-h)

Initially chalcone 4 was synthesised to build-up pyrazole ring and the synthetic pathway for the synthesis of chalcone 4 was shown in Scheme 1. Compound 2 was synthesized according to literature procedure, by the reaction of aldehyde 1 with 1,2-dibromoethane using Caesium carbonate as base and DMF as solvent. The base catalysed Claisen-Schmidt condensation of aldehyde 2 and 4-cyanoacetophenone 3 afforded chalcone4. The synthetic path way for the synthesis of title compounds 8(a-h) was represented in scheme-2. The oxidative cyclsation of chalcone 4 with N-phenyl hydrazine in the presence of molecular iodine in aq ethanol furnished compound 5 which on acid hydrolysis of nitirle group afforded pyrazole ester 6 in the presence of Ethanol-HCl. Hydrolysis of ethyl ester of compound 6 with Lithium hydroxide in aq THF followed by acidification produced scaffold compound 7. Finally the title compound 8(a-h) were synthesized by the reaction of scaffold 7 with corresponding amine 7(a-h), DIPEA in DCM via., formation of acid chloride with oxalylchloride.

Anticancer Activity

The calculated IC_{50} values of the newly synthesized compounds against human liver cancer cell line (Hep

G2) with reference to the drug substance "Sorafenib" were tabulated in Table 1. All the newly isolated compounds exhibiting anticancer activity. Among all the compounds, 8(h), 8(e), 8(d) exhibited high potentials and 8(f), 8(g) exhibiting moderate potentials of anticancer activity compared to the other compounds.

Antimicrobial Activity

The antimicrobial activity with the zone of inhibition of all the title compounds 4, 5, 6, 7 and 8(a-h) is presented in Table 2 at various concentration levels 200 μg, 300 μg and 500 μg. All the title compounds exhibiting antimicrobial activity but less activity than the reference drug "Streptomycin". Among all the title compounds, compounds 8(e), 8(f), 8(b), 8(d), 8(c)exhibiting good antibacterial activity against *Salmonella enterica*; compounds 4, 8(a), 8(h) exhibiting good antibacterial activity against *pseudomonas aureoginosa*; compounds 8(c), 8(f), 8(e), 8(b), 8(d) exhibiting good antibacterial activity against *Bacillus cerus*; compounds 8(h), 8(g), 8(e), 8(f), 6 exhibiting good antibacterial activity against *Streptococcus Pyrogenes* and title compounds 8(e), 8(h), 8(d), 5 exhibiting good antifungal activity against *Aspergillus niger*.

Table 1: *In vitro* cytotoxic activity of compounds 4,5,6,7 and 8 (a-h).

S.No	Compound	IC_{50}, μg/ml	S.No	Compound	IC_{50}, μg/ml
1.	4	80.21	7.	8(d)	46.5
2.	5	80.35	8.	8(e)	42.25
3.	6	83.25	9.	8(f)	59.35
4.	7	66.35	10.	8(g)	60.25
5.	8(a)	72.15	11.	8(h)	35.43
6.	8(b)	77.21	12.	Sorafenib	88.21

Antioxidant Activity

The ability of the free radical scavenging activity of all the newly synthesized compounds were studied using DPPH and ABTS assay methodologies with ascorbic acid as reference standard. The calculated IC_{50} values by both DPPH and ABTS methodologies were tabulated in Table 3. All the title compounds exhibiting variable antioxidant activities against both DPPH and ABTS with respect to ascorbic acid but less active than ascorbic acid. The results of DPPH assay methodology revealed that, compounds 4, 5, 8(h), 6, 8(c) demonstrated strong antioxidant activities when compared to the rest of the compounds. Further, the results derived using the methodology of ABTS assay are also supporting the strong antioxidant activity of the compounds 4, 5 8(h), 6, 8(c) as in the case of DPPH methodology.

Molecular Docking

Molecular docking of the newly isolated compounds has been carried out with protein BCL2. The binding efficiencies of the newly synthesized compounds were assessed by using their fitness values generated and are tabulated in Table 4. The binding modes of all the newly synthesized compounds with BCL2 were shown in Figure 3. Molecular docking results revealed that compounds 7, 8(f), 8(h) exhibiting very good binding efficiencies; compounds 5, 6, 4, 8(d) exhibiting moderate

Table 2: Antimicrobial activity of the newly synthesized compounds.

Compound	Gram (–) ve bacteria						Gram (+) ve bacteria						Fungi		
	Salmonella enterica			*Pseudomonas aureoginosa*			*Bacillus Cereus*			*Streptococcus pyrogenes*			*Aspergillus niger*		
	200	300	500	200	300	500	200	300	500	200	300	500	200	300	500
4	1.5	2.2	4.2	3.2	4.5	5.8	2.2	3.4	4.1	2.2	3.2	4.2	1.2	2.0	2.7
5	1.2	2.4	4.4	1.8	2.2	3.2	2.1	3.8	4.3	2.1	3.5	4.4	1.4	2.1	2.8
6	1.6	2.2	4.3	1.4	2.1	3.4	1.8	3.1	4.2	2.4	3.3	4.5	1.6	2.2	2.7
7	1.4	2.4	4.5	1.6	2.4	3.3	1.6	3.2	4.4	2.6	3.4	4.2	1.6	2.3	2.8
8(a)	1.3	2.7	4.4	2.8	3.2	4.6	2.4	3.4	4.6	2.3	3.1	4.1	1.4	2.2	2.3
8(b)	1.8	2.8	4.8	1.8	2.6	3.6	2.8	4.1	5.1	2.7	3.3	4.4	1.3	2.0	2.4
8(c)	2.2	2.9	4.6	1.7	2.7	3.3	2.3	4.3	5.3	2.5	3.4	4.2	1.2	2.1	2.5
8(d)	2.4	3.0	4.7	1.6	2.4	3.5	2.6	4.2	5.1	2.4	3.1	4.3	1.0	2.3	2.8
8(e)	2.8	3.2	5.4	1.5	2.3	3.2	1.9	4.7	5.2	2.6	3.4	4.8	1.2	2.2	2.9
8(f)	3.0	3.5	5.2	1.8	2.2	3.3	3.2	4.0	5.3	2.5	3.6	4.5	1.5	2.1	2.7
8(g)	3.5	3.8	3.5	1.9	2.8	3.6	1.8	2.9	3.2	3.2	4.3	5.4	1.2	2.1	2.8
8(h)	2.4	3.6	3.8	1.8	2.5	3.7	1.6	2.8	3.1	3.6	4.6	5.7	1.3	2.2	2.9
Streptomycin	6.8	7.5	8.2	7.3	8.8	9.6	8.0	8.5	9.5	8.5	9.8	10.2	3.0	4.0	5.0

Table 3: Antioxidant activity of newly synthesized compounds.

Compound	Radical-scavenging activity (IC_{50}, μg/mL)		Compound	Radical-scavenging activity(IC_{50}, μg/mL)	
	DPPH	ABTS		DPPH	ABTS
4	4.24 ± 0.26	3.46 ± 0.32	8(d)	8.25 ± 0.48	7.32 ± 0.21
5	5.35 ± 0.46	4.23 ± 3.43	8(e)	7.43 ± 0.56	6.25 ± 0.42
6	5.64± 0.43	4.52 ± 0.23	8(f)	8.25 ± 1.36	7.54 ± 0.35
7	7.38 ± 0.36	6.25 ± 0.15	8(g)	6.83 ± 0.48	5.26 ± 0.26
8(a)	8.24 ± 0.41	7.42 ± 0.36	8(h)	5.38 ± 0.53	4.43 ± 0.64
8(b)	7.43 ± 0.35	6.53 ± 0.42	Ascorbic acid (Vitamin C)	3.25 ± 0.15	2.42 ± 0.35
8(c)	6.62 ± 0.42	5.12 ± 0.38			

Table 4: Docking results of synthesized compounds.

S.No	Compound	Fitness	S(hb_ext)	S(vdw_ext)	S(hb_int)	S(int)
1.	4	34.90	0.00	31.08	0.00	-7.84
2.	5	33.25	0.00	28.28	0.00	-5.64
3.	6	34.58	5.88	26.06	0.00	-7.13
4.	7	30.24	0.00	25.63	0.00	-4.99
5.	8(a)	40.57	8.00	31.31	0.00	-10.49
6.	8(b)	40.07	7.47	31.29	0.00	-10.42
7.	8(c)	42.12	4.62	34.84	0.00	-10.40
8.	8(d)	36.41	8.00	28.84	0.00	-11.25
9.	8(e)	38.25	2.71	35.86	0.00	-13.77
10.	8(f)	32.17	2.63	31.14	0.00	-13.28
11.	8(g)	37.52	2.58	33.11	0.00	-10.58
12.	8(h)	32.30	6.00	28.73	0.00	-13.19

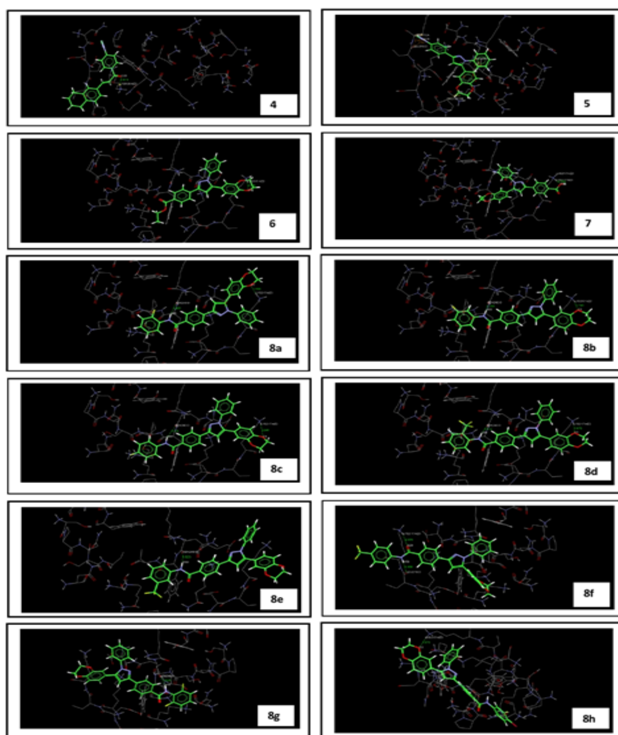


Figure 3: Binding models of synthesized componds with BCL2 protein.

binding efficiencies with the protein BCL2 compared to the rest of the compounds.

CONCLUSION

All the newly synthesized compounds exhibiting very good anticancer activity against liver cancer cell line (Hep G2). Among all the synthesized compounds, compounds 8(h), 8(e), 8(d) exhibited high potentials and compounds 8(f), 8(g) exhibited moderate potentials of anticancer activity compared to the rest of the compounds with reference to the drug substance "Sorafenib". All the synthesized compounds exhibiting variable antimicrobial activities and antioxidant activities with reference to "streptomycin" and "ascorbic acid" respectively. Further, the Molecular docking study revealed that, all the synthesized compounds exhibited very good binding efficiencies with protein BCL2. Among all the synthesized compounds, title compounds 7, 8(f), 8(h) exhibiting very good binding efficiencies and title compounds 5, 6, 4, 8(d), 8(g) exhibiting moderate binding efficiencies with protein BCL2 compared to the rest of the synthesized compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FTIR: Fourier-transform infrared spectroscopy; **NMR:** Nuclear magnetic resonance; **DMSO-*d*$_6$:** Dimethyl sulfoxide-*d*$_6$; **DMSO:** Dimethyl sulfoxide; **TMS:** Trimethylsilane; **ppm:** Parts per million; **Hz:** Hertz; **J:** Coupling constant; **ESI:** Electrospray ionisation; **API:** **A**tmospheric-pressure chemical ionisation; **UV:** Ultra violet; **TLC:** Thin layer chromatography; **DMF:** N, N-Dimethylformamide; **M.P:** Meltingpoint; **DCM:** Dichloromethane; **GOLD:** Genetic Optimization of Ligand Docking; **BCL2:** B-cell lymphoma 2; **DPPH**: Diphenyl phosphoryl azide; **ABTS:** 2,2'-azino-bis(3-ethyl benzo thiazoline-6-sulfonic acid).

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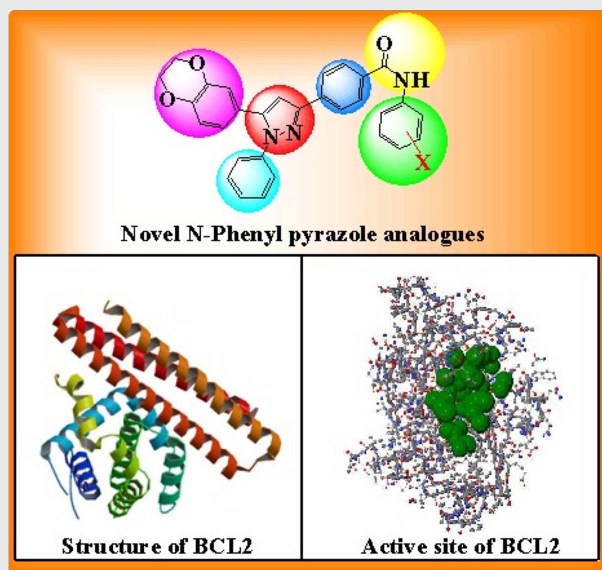
SUMMARY

A novel series of N-Phenyl derivatives 8(a-h) were prepared and then characterized by modern instrumental techniques viz., ^{1}H NMR, ^{13}C NMR, Mass and FTIR. The newly synthesized compounds screened for anticancer, antimicrobial and antioxidant activity. Among all the title compounds, Compounds 8(h), 8(e), 8(d) exhibited high potentials and 8(f), 8(g) exhibited moderate potentials of anticancer activity when compared to the rest of the compounds with reference to with "Sorafenib". Compounds 8(e), 8(f), 8(b), 8(d), 8(c)exhibiting good antibacterial activity against *Salmonella enterica*; compounds 4, 8(a), 8(h) exhibiting good antibacterial activity against *pseudomonas aureoginosa*; compounds 8(c), 8(f), 8(e), 8(b), 8(d) exhibiting good antibacterial activity against *Bacillus cerus*; compounds 8(h), 8(g), 8(e), 8(f), 6 exhibiting good antibacterial activity against *Streptococcus Pyrogenes* and title compounds 8(e), 8(h), 8(d), 5 exhibiting good antifungal activity against *Aspergillus niger* with reference to "Streptomycin". All the title compounds exhibiting variable antioxidant activities against both DPPH and ABTS with respect to ascorbic acid but less active than ascorbic acid. The results of DPPH assay methodology revealed that, compounds 4, 5, 8(h), 6, 8(c) demonstrated strong antioxidant activities when compared to the rest of the compounds. Further, the results derived using the methodology of ABTS assay are also supporting the strong antioxidant activity of the compounds 4, 5 8(h), 6, 8(c) as in the case of DPPH methodology. Molecular docking of the synthesized compounds with BCl2 protein revealed that compounds 7, 8(f), 8(h) exhibiting very good binding efficiencies; compounds 5, 6, 4, 8(d) exhibiting moderate binding efficiencies with the protein BCL2 compared to the rest of the compounds.

PICTORIAL ABSTRACT



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