2. EXPERIMENTAL

All chemicals used are of analar grade. The melting points are determined using open capillaries and are uncorrected.

2.1. General procedure for the synthesis of thiazoles

2.1.1. \((Z)-4-\text{phenyl}-2-((1-\text{phenylethylidene})\text{hydrazinyl})\text{thiazoles (46–55)}\)

Equimolar amount of aryl ketones (0.1 mmol), thiosemicarbazide (0.1 mmol) and substituted phenacyl bromide (0.1 mmol) were mixed and subjected to microwave irradiation for 30–55 s at a heating rate of 300 W. After the reaction has completed, the reaction mixture was taken out, the solid product is recrystallized from ethanol to get pure compounds (Scheme 35 and 36).

2.1.2. \(2-((2-\text{(diphenylmethylene)hydrazinyl})-4-\text{phenylthiazoles (56–63)})\)

Equimolar mixture of biaryl ketones (0.1 mmol), thiosemicarbazide, (0.1 mmol) and substituted phenacyl bromide (0.1 mmol) were mixed and subjected to microwave irradiation for 30–175 s at a heating rate of 300 W. After the reaction has completed, the reaction mixture was taken out and the solid product was recrystallized from ethanol (Scheme 37).

2.1.3. \((Z)-4-\text{phenyl}-2-((2-(\text{pyridin-4-y(or)pyridin-2-y)methylene})\text{hydrazinyl})\text{thiazoles (64–68)})\)

Equimolar quantities of 4-benzoyl pyridine thiosemicarbazone (0.1 mmol) and substituted phenacyl bromide(0.1 mmol) were mixed and must be subjected to microwave irradiation for 30–120 s at a heating rate of 400 W. After the completion of reaction it was taken out and cooled to room temperature. The solid crude product was washed with acetonitrile to get pure compounds (Scheme 38).
2.1.4. \((Z)-1-((2-(4-phenylthiazol-2-yl)hydrazono)methyl)naphthalene-2-ols\) \(69\) and \(70\)

Equimolar mixture of 2-hydroxy naphthaldehyde (0.1 mmol), thiosemicarbazide (0.1 mmol) and substituted phenacyl bromide (0.1 mmol) were mixed and subjected to microwave irradiation for 30–175 s at a heating rate of 300 W. After the reaction has completed reaction mixture was taken out and the solid product was recrystallized from ethanol to get pure compounds (Scheme 39).

2.2. General procedure for the synthesis of highly functionalized piperidines.

2.2.1. Ethyl 1,4-dibenzyl-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylates (71–79) and Ethyl 1,2,6-triphenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylates (80 and 81)

A mixture of aliphatic (or) arylamines (2.0 mmol), arylaldehyde (2.0 mmol) and ethyl acetoacetate (1.0 mmol) in the presence of tetrabutylammonium bromide and iodine (TBAB/I\(_2\)) in 25 mL of ethanol was stirred for 5–7 h at room temperature. After completion, the reaction mixture was poured into crushed ice. The colored product were extracted with DCM (Scheme 40) and subjected to column chromatography using hexane and ethyl acetate (9:1) as eluent. The pure products were characterized by conventional spectroscopic methods.

2.3. General procedure for the synthesis of 3-alkyl indoles.

2.3.1. 3-\{(phenyl(phenylamino)methyl)-1H-indole-5-carbonitriles\} (82–86)

A mixture of aryl amines (2.0 mmol), aryl aldehyde (2.0 mmol) and 5-cyano indole (2.0 mmol) were stirred in a preheated oil bath at 120 °C for the appropriate time of (7–9 h) Scheme 41. The progress of the reaction was monitored by thin-layer
chromatography (TLC). After completion, the reaction mixture was poured into crushed ice. The precipitate was filtered, washed with water and dried. The crude product was then subjected to column chromatography using hexane and ethyl acetate (3:1) as eluent to get pure product.

2.3.2. 3,3′-(phenylmethylene)bis(1H-indole-5-carbonitrile) (87 and 88)  

Aryl aldehyde (2.0 mmol) with 5-cyano indole (2.0 mmol) was stirred in a preheated oil bath at 120 °C for the appropriate time of (6–8 h) Scheme 41. The progress of the reaction was monitored by thin-layer chromatography (TLC). After completion, the reaction mixture was poured into crushed ice. The precipitate was filtered, washed with water and dried. The crude product was then subjected to column chromatography using hexane and ethylacetate (3:1) as eluent to get pure product.

2.4. General procedure

2.4.1 Synthesis of 3-acetyl-2H-benzo[g]chromen-2-one (89)  

To the mixture of 2-hydroxy naphthaldehyde and ethyl acetoacetate piperidine was added drop wise and stirred for 3–5 h Scheme 42. The reaction mixture was left overnight, resulting in the formation of a yellow coloured solid product. Purification by recrystallized in ethanol gave 3-acetyl-2H-benzo[g]chromen-2-one (89) as yellow crystal.

2.4.2. Synthesis of hydrazinyl carbothioamide derivatives (90–92)  

Equimolar mixture of ketones, thiosemicarbazide and substituted phenacyl bromide were mixed and subjected to microwave irradiation for 50–60 s at a heating rate of 320 W Scheme 42. After the reaction has completed the reaction mixture
was taken out, the solid product was recrystallized from ethanol to get pure compounds.

2.5. EXPERIMENTAL SECTION

2.5.1. General

All the reported melting points were recorded in open capillaries and were uncorrected. The IR spectrum was recorded in AVATAR–330 FT–IR spectrophotometer and only noteworthy absorption levels (reciprocal centimeters) were listed. $^1$H NMR spectra were recorded at 400 and 500 MHz on a Bruker AMX 400 and 500 MHz spectrophotometers using CDCl$_3$ as solvent and TMS as an internal reference; chemical shifts ($\delta$ scale) are reported in parts per million (ppm). $^1$H NMR spectra are reported in the order: no of hydrogens, multiplicity and coupling constant ($J$ value) in hertz (Hz); signals were characterized as an s (singlet), d (doublet), t (triplet), m (multiplet), bs (broad singlet). $^{13}$C NMR spectra were recorded at 100 and 125 MHz on a Bruker AMX 400 and 500 MHz spectrophotometer using CDCl$_3$ as the solvent. DEPT–135 spectrum was recorded at 100 MHz on a Bruker AMX 400 MHz spectrophotometer using CDCl$_3$ as the solvent. $^1$H–$^1$H COSY and $^1$H–$^{13}$C COSY spectra were recorded on Bruker AMX 400 NMR spectrometer using standard parameters and 0.05 M solutions of the sample prepared using CDCl$_3$ were used for recording 2D NMR spectra. The tubes used for recording NMR spectra were 5 mm in diameter. HRMS (ESI) were carried out in a Bruker Maxis instrument at the School of Chemistry, University of Hyderabad. Elemental analyses (CHN) were carried out on a Thermo Finnigan Flash EA 1112 analyzer at the School of Chemistry, University of Hyderabad. Routine monitoring of the reactions was performed by TLC, using silica gel plates (Merck 60 F254) and compounds were visualized with a UV light at 254 nm. Crystal data were collected
with Oxford and Bruker Smart Apex–II CCD diffractometers using graphite monochromated Mo–Kα radiation (λ= 0.71073 Å) at 298 K.

### 2.5.2. SPECTRAL MEASUREMENTS

#### 2.5.2.1. FT–IR SPECTRA

The spectra were recorded on AVATAR–330 FT–IR spectrophotometer (Thermo Nicolet) and only noteworthy absorption levels (reciprocal centimeters) are listed. FT–IR spectra were recorded on a JASCO FT–IR 5300 spectrometer; absorptions are reported in cm\(^{-1}\).

#### 2.5.2.2. \(^1\)H NMR SPECTRA

\(^1\)H NMR spectra were recorded on BRUKER AMX–400 and 500 spectrometer operating at 400 and 500 MHz. Samples were prepared by dissolving 50 mg of compounds in 0.5 ml of DMSO–d\(_6\), CDCl\(_3\) 1% TMS. The entire chemical shift is reported to TMS.

#### 2.5.2.3. \(^{13}\)C NMR Spectra

The \(^{13}\)C NMR spectra were recorded on BRUKER AMX–400 or 500 spectrometer operating at 100 or 125 MHz. Samples were prepared by dissolved 50mg of the compound in 0.5 ml of DMSO–d\(_6\) or CDCl\(_3\) 1% TMS.

#### 2.5.2.4. DEPT SPECTRA

The DEPT spectra were recorded on BRUKER AMX–400 spectrometer operating at 100 MHz. Samples were prepared by dissolved 50 mg of the compound in 0.5 ml of CDCl\(_3\) 1% TMS.

#### 2.5.2.5. HOMO COSY and HETERO SINGLE QUANTUM CORRELATION SPECTRA

The \(^1\)H–\(^1\)H and \(^1\)H–\(^{13}\)C COSY spectra were recorded on a BRUKER AMX–400 spectrometer operating samples were prepared
by dissolving 50 mg of the compound in 0.5 ml of DMSO–d₆ or CDCl₃ 1% TMS.

2.5.2.6. MASS SPECTRA

Mass spectra were recorded on either a VG7070H mass spectrometer using an EI technique, or a Shimadzu–LCMS–2010A mass spectrometer and HRMS (ESI) was carried out in a Bruker Maxis instrument samples were prepared by dissolving about 1 mg of compound in 2 ml of spectral grade methanol.

2.5.2.7. ELEMENTAL ANALYSIS.

Elemental analyses (CHN) were recorded on a Thermo Finnegan Flash EA 1112 analyzer at the School of Chemistry, University of Hyderabad.

2.5.3. X–RAY SINGLE CRYSTALLOGRAPHIC

X–Ray diffraction measurements were carried out at 298 k on an automated diffractometer using graphite–monochromated Mo–Kα (λ = 0.7107 Å) radiation with CAD4 software or the X–ray intensity data were measured at 298 k equipped with a graphite monochromator and a Mo–Kα fine–focus sealed tube (λ = 0.71073 Å).

2.6. BIOLOGICAL ACTIVITY

2.6.1. Antibacterial activity

The following Gram–positive and Gram–negative strains have been used for the study.

1. *Escherichia coli* (Gram–negative)
2. *Salmonella typhi* (Gram–negative)
3. *Staphylococcus aureus* (Gram–positive)
4. *Klebsiella pneumoniae* (Gram–positive)
5. *Pseudomonas aeruginosa* (Gram–positive)
Preparation of test inoculums

a) Sub-culture (preparation of seeded broth)

The strains of *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were inoculated in conical flasks containing 100 mL of sterile nutrient broth. These conical flasks were incubated at 37±1°C for 24 h. This was used as seeded broth.

b) Standardization of seeded broth (viable count)

Dilutions

1 mL of seeded broth of each strain was diluted with 99 mL of sterilized normal saline containing 0.05% tween 80 (8 drops of tween 80 in 1000 mL of normal saline). From that, 1 mL was further diluted to 10 mL with sterile normal saline. This was continued to until 10⁻¹⁰ mL of the dilution of seeded broth was obtained.

Incubation of nutrient agar petridishes

The dilutions were studied by inoculating 0.2 mL of each dilution on to the solidified nutrient agar medium by spread plate method after incubation at 37±1°C for 24 h. The number of well-formed colonies on the plates was counted. The seeded broth was then suitably diluted to have between 10⁵–10⁷ microorganisms per millimeter or cfu/mL. This was designated as the working stock and used for the antibacterial studies.

Preparation of solution of test compounds

The solution of test compounds were prepared by dissolving the same in dimethylsulfoxide (DMSO) in specific gravity bottle and stored in refrigerator. The solution was removed from the refrigerator 1 h prior to its use and allowed to warm up to the room
temperature. The test compounds were prepared at a concentration of 200 µg/mL. Similarly, the standard drug solutions of Amikacin and Amphotericin B were used respectively at a concentration of 200 µg/mL for finding the minimum inhibitory concentration. Solvent control of DMSO was also maintained throughout the experiments simultaneously.

c) Preparation of culture media

The following mediums were used for the bacterial growth:

i) Nutrient agar medium

ii) Nutrient broth medium

The media were sterilized by autoclaving at a pressure of 15 lb/sq at 121°C for 20 min.

i) Nutrient agar medium (Hi-Media)

The nutrient agar medium was prepared by dissolving 28 g of nutrient agar (procured from Hi-Media, Mumbai) in 1000 mL of distilled water.

Materials

- Peptone: 1%
- Sodium chloride: 0.5%
- Beef extract: 1%
- Agar: 2%
- pH: 7.4±0.2

ii) Nutrient broth medium (Hi-Media)

The nutrient broth medium was prepared by dissolving 13 g of nutrient broth (Hi-Media, Mumbai) in 1000 mL of distilled water.
Materials

Peptone : 1%
Sodium chloride : 0.5%
Beef extract : 1%
pH : 7.4±0.2

d) Determination of antibacterial activity by disc-diffusion method

Base plates were prepared by pouring 10 mL of autoclaved Muller-Hinton agar into sterile petridishes (9 cm) and allowing them to settle. Sterile blank discs (6 mm) were impregnated with 15 μL of known concentration of stock solution of tested complexes as to obtain discs containing 100 and 400 μg of each compound. Impregnated discs were air dried and cautiously placed on the surface of Mueller-Hinton agar plates freshly inoculated with microorganisms. After 10 min at room temperature the plated culture incubated for 24 h at 37°C. Experiments were conducted in quadruplicate (four discs with identical concentration of the same compound) and commercial antibiotic Amikacin (100 μg) impregnated discs used as positive controls. Susceptibility diameter zone was reported as the average value of replicates measurements.

2.6.2. Antifungal activity

The following fungal strains were used for the study.

1. Aspergillus flavus
2. Candida albicans
3. Aspergillus fumigatus
4. Aspergillus niger
a) Preparation of culture media

Sabouraud’s dextrose agar (SDA) medium was used for the growth of fungi and testing was done in Sabouraud’s dextrose broth (SDB) medium.

b) Antifungal disc diffusion method

Mature conidia of fungal isolates were harvested from potato dextrose agar (PDA) plates and suspended in ringer solution and spore suspensions standardized with a haemocytometer (10^4 conidia mL^-1). Conidial suspension (1 mL) representing each fungal isolate was then spread on a 9 cm petridishes containing PDA (20 mL) with the excess of conidial suspension decanted and allow to dry. The compounds were dissolved in dimethyl sulphoxide (DMSO). Sterile 6 mm diameter test discs were impregnated with 15 μL of the solution of each test compound to certain 100 and 400 μg/disc in triplicates. Amphotericin B was used as a reference drug, for fungal inhibition. While DMSO was used as a negative control. Plates were incubating at room temperature (22–25°C) for 3 days. The radius of the inhibition zone of fungal growth was measured after 3 days. Diameter zone was reported.

Preparation of culture media

(a) SDA medium

Formula

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<th>Amount</th>
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<tr>
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<td>1000g</td>
</tr>
<tr>
<td>pH</td>
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</table>
(b) SDA medium

Formula

<table>
<thead>
<tr>
<th>Ingredient</th>
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<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.3±0.2</td>
</tr>
</tbody>
</table>

2.7. MOLECULAR DOCKING STUDIES

The docking studies were conducted using the AutoDock 4.2, Accelrys Software Inc., 3.5, 2012. The PyMOL Molecular Graphics System, version 1.5.0.4. Schrödinger, LLC, Newyork 2012. The 3D co-ordinates of crystallographic structure of the lactoperoxidase with a potent inhibitor amino-triazole and BRCT (PDB ID: 3KRQ and 1YWN) was downloaded from Brookheaven protein Data Bank (www.rscb.com). Docking comprises five steps: protein preparation, ligand preparation, receptor grid generation, actual docking procedure, and viewing the docking results using the pose-viewer.

Molecular docking trials for compounds \(46, 48, 54-56, 65, 68-70\) and \(90-92\) were performed using the AutoDock4.2 program,\(^{138}\) this is one of the most widely used docking engines and its scoring function for hydrogen bond directionality\(^{139}\) is exploited in this study. The Lamarkian Genetic Algorithm (LGA) was used for the docking calculation. One hundred runs with 25000,000 (maximum) evaluations and 270,000 generations were used for the docking simulation. After comparing the binding modes of the best docked poses derived from each simulation with the corresponding cocrystal structure of a ligand, the poses with the root-mean-
square-deviation (rmsd) below 2.0 Å from crystal poses were chosen for further analysis.

For compounds (71-86) Molecular docking was performed by Glide module implemented in Maestro version 9.3.5 of Schrödinger software suite, 2011. The ligands were prepared by Ligprep application in which the conformers were generated using a rapid torsion angle search approach followed by minimization of each generated structure using the OPLS-2005 (Optimized Potential for Liquid Simulations) force field. The 3D co-ordinates of crystallographic structure of the 4-amino-fluro [2,3-d] pyrimidine PDE5A1 (PDB ID: 1YWN, 2H44) was downloaded from Brookheaven protein Data Bank (www.rscb.com). The protein complex was pre-processed and prepared by Protein Preparation Wizard in Maestro of Schrödinger. The minimization of the complex was continued using OPLS-2005 force field until the root mean square deviation (RMSD) reached the value of 0.3 Å. The molecular docking studies of the ligands and the protein were performed by GLIDE. Glide provides three different level of docking precision (HTVS, high throughput virtual screening; SP, standard precision, and XP, extra precision). We carried out our calculations in XP mode. The best fit molecules with the protein were ranked based on the G score.