ANTIMICROBIAL ACTIVITY

The compounds 2-((4-chloro-6-methylpyrimidin-2-ylthio)methyl)benzoxazole (50), 2-((4-chloro-6-methylpyrimidin-2-ylthio)methyl)benzothiazole (51), 2-((4-chloro-6-methylpyrimidin-2-ylthio)methyl)-1H-benzimidazole (52), 2-(2-((benzoxazol-2-yl)methylthio)-6-methylpyrimidin-4-ylthio)benzoxazole (53), 2-(2-((benzothiazol-2-yl)methylthio)-6-methylpyrimidin-4-ylthio)benzothiazole (54), 2-(2-((1H-benzimidazol-2-yl)methylthio)-6-methylpyrimidin-4-ylthio)-1H-benzimidazole (55), 2-(4-((benzoxazol-2-yl)methylthio)-6-methylpyrimidin-2-ylthio)methyl)benzoxazole (56), 2-(4-((benzothiazol-2-yl)methylthio)-6-methylpyrimidin-2-ylthio)methyl)benzothiazole (57), 2-(4-((1H-benzimidazol-2-yl)methylthio)-6-methylpyrimidin-2-ylthio)methyl)-1H-benzimidazole (58), N-(2-((benzoxazol-2-yl)methylthio)-6-methylpyrimidin-4-yl)benzoxazol-2-amine (59), N-(2-((benzothiazol-2-yl)methylthio)-6-methylpyrimidin-4-yl)benzothiazol-2-amine (60) and N-(2-((1H-benzimidazol-2-yl)methylthio)-6-methylpyrimidin-4-yl)-1H-benzimidazol-2-amine (61) (Chart) were assayed for antimicrobial activity.
Methodology:

The methodology used to study the antimicrobial activity is described in section I.

Microorganisms:

The following microorganisms were used to test the antimicrobial activity.

Bacteria:

- **Gram-positive**: 1. *Staphylococcus aureus*
- **Gram-negative**: 1. *Escherichia coli*
  2. *Pseudomonas aeruginosa*

Fungi:

1. *Aspergillus niger*
2. *Penicillium chrysogenum*

Determination of Minimum Inhibitory Concentration:

Broth dilution test is used to determine Minimum Inhibitory Concentration (MIC) of the above mentioned samples. Freshly prepared nutrient broth was used as diluents. The 24 and 48 hrs old culture of the test bacteria *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* and the test fungi *Aspergillus niger* and *Penicillium chrysogenum* were diluted 100 folds in nutrient broth (100 µl bacterial cultures in 10 ml NB). The stock solution of the synthesized compounds was prepared in DMSO by dissolving 5 mg of the compound in 1 ml of DMSO. Increasing concentrations of the test samples (1.25, 2.5, 5, 10, 20, 40 µl of stock solution contains 25, 50, 100 µg/well of the compounds) were added to the test tubes containing bacterial and fungal cultures. All the tubes were incubated at 37°C for 24 hrs for bacteria and at 28°C for 48 hrs for fungi. The tubes were examined for visible turbidity by using NB as control. Control without test samples and with solvent was assayed simultaneously. The lowest concentration that inhibited visible growth of the tested organisms was recorded as MIC (Table III.10).

Determination of Minimum Bactericidal / Fungicidal Concentrations:

To determine the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) for each set of test tubes in the MIC determination, a loopful
of broth was collected from those tubes which did not show any growth and inoculated on sterile nutrient broth (for bacteria) and PDA (for fungi) by streaking. Plates inoculated with bacteria and fungi were incubated at 37°C for 24 hrs and at 28°C for 48 hrs, respectively. After incubation, that concentration was noted as MBC (for bacteria) or MFC (for fungi) at which no visible growth was observed (Table III.10).

Result and discussion:

The compounds 50-61 were screened for antimicrobial activity at three different concentrations 25, 50, and 100 µg/well. The results of antibacterial activity presented in Table III.8 and Fig. III.11 indicated that all the tested compounds exhibited more activity towards Gram-positive bacteria than Gram-negative bacteria. The 4-chloropyrimidinylsulfanylmethyl benzoazole (50), benzothiazole (51) and benzimidazole (52) displayed least activity. Further, the amino linked heterocycles 59, 60 and 61 showed slightly higher activity than those having thio group 53, 54 and 55. Replacement of chloro substituent by heterocyclic moiety enhanced the activity. Amongst tris heterocyclic compounds, pyrimidinyl bis methylthio benzoazole (56), benzothiazole (57) and benzimidazole (58) displayed greater activity. In fact, compound 58 showed activity higher than the standard Ciprofloxacin at all tested concentrations towards Staphylococcus aureus.

All the tested compounds inhibited the spore germination against tested fungi. In general most of the compounds showed slightly higher antifungal activity towards Penicillium chrysogenum than Aspergillus niger. The compound 58 displayed excellent activity particularly against Penicillium chrysogenum equivalent to the standard drug Ketoconazole at 100 µg/well (Table III.9 and Fig. III.12).

The minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal concentration (MFC) values of the compounds tested are listed in Table III.10. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. (However it is not sure that the microorganisms are completely killed). The MBC/MFC is the lowest concentration of antibiotic required to kill a particular bacterium/fungi. The MBC/MFC involve an additional set of steps performed once the minimum inhibitory concentration (MIC) is
determined. The antimicrobials are usually regarded as bactericidal/fungicidal if the MBC/MFC is not greater than four times the MIC. The compound 58 exhibited low MIC values when compared with 56 and 57. In addition MBC value is 2 x MIC in case of Staphylococcus aureus and MFC value is 2 x MIC in case of Penicillium chrysogenum. However, the other compounds showed bactericidal and fungicidal effects greater than 2 x MIC. The structure-antimicrobial activity relationship of the tested compounds indicated that tris heterocyclic compounds separated by methylthio moiety (56, 57 & 58) exhibited greater activity than bis heterocyclic compounds (50, 51 & 52) and tris heterocyclic compounds having amino (59, 60 & 61) and thio (53, 54 & 55) moieties. Amongst those having methylthio unit, the benzimidazole containing compound 58 exhibited excellent activity against Staphylococcus aureus with an inhibition zone of 29 mm at 100 µg/well and MIC and MBC of 12.5 and 25 µg/well, respectively. The compound 58 also displayed strong antifungal activity against Penicillium chrysogenum with an inhibition zone of 38 mm at 100 µg/well and MIC and MFC of 12.5 and 25 µg/well, respectively.

**TABLE III.8.**

The *in vitro* antibacterial activity of compounds 50-61.

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>25 µg/well</td>
<td>50 µg/well</td>
</tr>
<tr>
<td>50</td>
<td>05±3</td>
<td>07±3</td>
</tr>
<tr>
<td>51</td>
<td>06±2</td>
<td>04±1</td>
</tr>
<tr>
<td>52</td>
<td>08±2</td>
<td>10±3</td>
</tr>
<tr>
<td>53</td>
<td>09±4</td>
<td>11±2</td>
</tr>
<tr>
<td>54</td>
<td>10±2</td>
<td>12±3</td>
</tr>
<tr>
<td>Compd. No.</td>
<td>Zone of inhibition (mm)</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------------</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>A. niger 25µg/well</td>
<td>50µg/well</td>
</tr>
<tr>
<td>55</td>
<td>13±2</td>
<td>16±2</td>
</tr>
<tr>
<td>56</td>
<td>20±2</td>
<td>22±2</td>
</tr>
<tr>
<td>57</td>
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<td>23±1</td>
</tr>
<tr>
<td>58</td>
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<td>26±1</td>
</tr>
<tr>
<td>59</td>
<td>10±3</td>
<td>13±2</td>
</tr>
<tr>
<td>60</td>
<td>13±2</td>
<td>16±1</td>
</tr>
<tr>
<td>61</td>
<td>15±3</td>
<td>17±2</td>
</tr>
</tbody>
</table>

Ciprofloxacin Control (DMSO)  
22±1 24±3 27±1 30±2 35±3 38±2 25±1 28±2 30±3

(-) No activity  
(±) Standard deviation

**TABLE III.9.**  
The *in vitro* antifungal activity of compounds 50-61.
<table>
<thead>
<tr>
<th>Compd.</th>
<th>Minimum inhibitory concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>MIC (MBC/MFC) mg/well</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>56</td>
<td>50 (&gt;200)</td>
</tr>
<tr>
<td>57</td>
<td>50 (200)</td>
</tr>
<tr>
<td>58</td>
<td>12.5 (25)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>12.5</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>-</td>
</tr>
</tbody>
</table>

MIC, MBC and MFC of compounds 56, 57, and 58.

(-) No activity
Fig. III.11 The \textit{in vitro} antibacterial activity of compounds 50-61.

![Graph showing antibacterial activity of compounds 50-61](image)

Fig. III.12 The \textit{in vitro} antifungal activity of compounds 50-61.

![Graph showing antifungal activity of compounds 50-61](image)
Cytotoxic activity:

Cultures of A549 cells:

The compounds 50-61 were dissolved in DMSO and used for making different concentrations of dilutions 12.5-200 µM to treat cells.

Lung adenocarcinoma cells (A549) were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium) substituted with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were cultured in T-25 flask. These cultured cells were plated in 96 well tissue culture plates and incubated at 37 °C in a 5% CO₂ atmosphere with 90% humidity for cytotoxicity studies.

(3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (MTT) assay for cell viability:

The cytotoxicity of the compounds was tested on A549 lung carcinoma cells in a 96-well tissue culture cluster (Nunc Inc Germany) plated with 5x10⁴ cells in a each well and incubated at 37°C in a medium containing DMEM, 10% fetal bovine serum and antibiotics (Invitrogen, USA), in a 5% CO₂ atmosphere. After attachment and 60% confluent of the cells (usually 24 hrs), different concentrations of each compound were added and incubated for 20 hrs. MTT solution (20 µl of 5 mg/ml) was added to each well and the incubation continued for additional 3 hrs. The dark blue formazan crystals formed within the healthy cells were solubilized with DMSO and the absorbance was estimated in ELISA plate reader (7520 Micro plate reader, Cambridge technologies, Inc) at 550 nm and the absorbance was correlated with the cell number. Experiments were performed in triplicate and the values are the average of three (n = 3) independent experiments. The inhibitory concentration (IC₅₀) of the compound was assessed by Graph Pad Prism software.

Results and Discussion:
The compounds 50-61 were subjected to MTT assay to determine growth inhibitory/cytotoxic capability. The compounds 54 and 60 showed cytotoxic activity on A549 cells with IC$_{50}$ values of 70 $\mu$M and 10.5 $\mu$M, respectively. However, the remaining compounds did not display any cytotoxicity when used up to 200 $\mu$M concentration. Figs. III.13 and III.14 shows the results of cytotoxicity of 54 and 60 using MTT assay on A549 lung adenocarcinoma cells. The A549 cells have a mutated K-ras oncogene. The cytotoxic activity observed with compounds 54 and 60 is concentration dependent. Compound 60 at concentrations 12.5-200 $\mu$M showed lowest viability, while viability more than 50% is observed when this compound is used at a concentration below 12.50 $\mu$M on A549 cells. This suggests that compound 60 as a noticeable lead molecule for cytotoxic activity against tumor cells.

**Fig. III.13** The dose response curve of 54 measured by MTT assay on A549 lung adenocarcinoma cells.

![MTT Assay on A549@24h with vptr1compound](image)

IC$_{50}$=70$\mu$M

**Fig. III.14** The dose response curve of 60 measured by MTT assay on A549 lung adenocarcinoma.

![MTT Assay on A549@24h with RC1Compound](image)

IC$_{50}$ = 10.5$\mu$M