CHAPTER 2  
EXPERIMENTAL TECHNIQUES

2.1 INTRODUCTION

Details of experimental techniques and sophisticated instruments used for the structural study of all the newly synthesized compounds have been included in this chapter. The chapter also includes protocols for the biological activities discussed in chapter number 3, 4, 5 and 6 against insects and microorganisms.

2.2 ELEMENTAL ANALYSIS

The elemental analyses (C, H, N and S) of representative synthesized derivatives were performed on Thermo Finnigan Flash EA 1112 series CHNS (O) analyzer at Indian Institute of Technology, Powai, Mumbai (India). The theoretical % of C, H, N and S elements were calculated on Chem Draw Chemistry software and compared with the practical values. The data has been included under each series and discussed in respective chapters.

2.3 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The \(^1\text{H}\) and \(^{13}\text{C}\) Nuclear Magnetic Resonance spectra were scanned at University of Pune, Pune on a 300 MHz Varian Mercury YH-300 FT NMR in CDCl\(_3\) (7.26 delta - solvent residual signals, 1-2 delta- water) and DMSO-d\(_6\) (2.50 delta- solvent residual signals, 3.33 delta - water) using TMS as an internal standard and interpreted data of each series are discussed in respective chapters. Chemical shifts are reported in \(\delta\) values relative to TMS.

2.4 MASS SPECTROMETRY

The mass spectra of representative synthesized derivatives were recorded from SAIF, Indian Institute of Technology, Powai, Mumbai (India) on Shimadzu LCMS-QP8000 LC MS spectrometer. The molecular weights (theoretical and practical) were calculated on Chem Draw Chemistry software and data have been interpreted and discussed for each series in respective chapters.

2.5 INFRARED (IR) SPECTROSCOPY
FT-IR spectra were recorded as Nujol mulls in the wave number range 4000-400 cm$^{-1}$ on Perkin Elmer Spectrum one, FTIR Spectrophotometer at the School of Chemical Sciences, North Maharashtra University, Jalgaon (India) and the interpreted data of each series have been discussed in respective chapters.

### 2.6 MELTING AND BOILING POINTS

After their crystallization and proper drying of synthesized compounds, their melting points were determined by open capillary method by using Thieles tube while the boiling points were also determined by using Thieles tube by Siwoloboff’s method and are uncorrected for pressure effects. The results are presented in respective chapters.

### 2.7 R$_f$ VALUES

Thin Layer Chromatography (TLC) was performed on 200 µm thick aluminium sheets having silica gel 60 F$_{254}$ as an adsorbent. The different proportion systems of polar and non-polar solvent were used for developing the TLC plates. The purity of synthesized compounds were checked by using thin layer chromatography. UV-light and iodine were used for the visualization of the spots.

The $R_f$ values were calculated by formula given below:

$$R_f = \frac{\text{Distance moved by the substance}}{\text{Distance moved by the solvent front}}$$

### 2.8 SINGLE CRYSTAL X-RAY CRYSTALLOGRAPHY

The structures were solved by direct methods and refined with full matrix least-squares technique. X-ray diffraction data were collected on an Oxford Xcalibur Eos Mova diffractometer equipped with a CCD detector utilizing MoKα radiation ($\lambda = 0.71073$ Å). All calculations were performed using the WinGX software package. Diffractometer studies were carried out at Solid State and Structural Chemistry Unit, Indian Institute of Science, Bangalore (Karnataka, India). Structures of the molecules are described in the respective chapters.

### 2.9 BIOLOGICAL ACTIVITY

#### 2.9.1 Insect Growth Regulator Activity:}

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All the synthesized compounds (Schemes 1, 2, 3 and 4) were evaluated for insect growth regulator (IGR) activity on the Agricultural pest *Spodoptera litura* and compounds of Scheme 1 were also evaluated for IGR activity on red cotton bug. The detailed bioassay is discussed in this section and the results are discussed in Chapters 3, 4, 5 and 6.

2.9.1.1 Rearing of Test Insect *S. litura*

Culture of *Spodoptera litura* was maintained at 25±10°C, 60±5% relative humidity and 16:8 hour photo : scotophase on artificial diet. An artificial diet of the composition given in Table 1 was used in the study. Kidney bean (*Phaseolus vulgaris*) seeds procured from market were washed thoroughly and soaked overnight in water. Soaked seeds were ground in an electric grinder carefully with the addition of 400 ml double distilled water. Wheat bran, wheat germ, ascorbic acid, casein, yeast powder, methyl p-hydroxybenzoate, sorbic acid, cholesterol, streptomycin sulphate, formaldehyde and multivitamin (ABDEC drops) were added to the ground material and mixed methodically. Agar was boiled in 200 ml double distilled water with constant stirring till it attained necessary consistency and then ground with the rest of the ingredients once again. The whole mixture was poured into plastic trays and covered with thin plastic film. After cooling, the diet was kept in refrigerator and used after 24 hours of ageing.

Neonates, upon hatching from egg, were transferred to plastic bottles (4 cm diameter and 1.5 cm high) containing thin slices of artificial diet. Five-day old larvae were transferred to plastic boxes (30 cm long, 20 cm wide and 7 cm high) containing pieces of diet in groups of twenty larvae. Trays were cleaned daily and larvae were fed with fresh diet. When the larvae exhibited gut purge and entered into nonfeeding wandering stage they were transferred to boxes containing saw dust for pupation. Pupae were collected after four to five days and disinfected with 0.02% sodium hypochlorite. Upon emergence of adults they were transferred to oviposition cages.

Adults were fed with 20% honey solution containing vitamin C, E and streptomycin sulphate. Castor leaves with their petiole dipped in water were provided for oviposition inside the cages. All the containers used for rearing were periodically disinfected with Protasan DS® (Qualigens). This enabled to maintain a disease-free and healthy stock culture for further experiments.
Larvae for experimental purposes were reared on washed and dried castor leaves in plastic trays. Care was taken to avoid overcrowding and strict hygiene was maintained to prevent any infection.

Table 2.1: Composition of semi – synthetic diet for *S. litura*

<table>
<thead>
<tr>
<th>Content</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney bean</td>
<td>65.00 g</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>55.00 g</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Casein</td>
<td>03.00 g</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>04.00 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>25.00 g</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>00.92 g</td>
</tr>
<tr>
<td>Methyl paraben</td>
<td>00.40 g</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>00.25 g</td>
</tr>
<tr>
<td>Agar</td>
<td>10.00 g</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>00.10 g</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>02.00 ml</td>
</tr>
<tr>
<td>Multivitamin (ABDEC) drops</td>
<td>03.00 drops</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>600.0 ml</td>
</tr>
</tbody>
</table>

2.9.1.2 Diet incorporation

In order to test the effect of chronic exposure of test compounds on *S. litura*, larvae were fed with artificial diet treated with derivatives. Test compounds at different concentration, such as 10 ppm, 100 ppm, 300 ppm, 500 ppm, 1000 ppm and 5000 ppm, were prepared in artificial diet according to Akhtar and Isman (2004). Control diets were prepared with the carrier solvent acetone alone. Test diets (258.56 gm) containing pure compounds were prepared as follows.

For a diet containing 0.01% (10 ppm,) fresh weight of the test substance, 25.856 mg of the substance was dissolved in 5 ml of the carrier solvent. This was pipetted on to the dry portion of the diet in a Petri dish. The solvent was allowed to evaporate. This was mixed well with 140 ml of double distilled water in an electric blender. 3.3 g of agar was dissolved in 60 ml of double distilled water and heated to boil. Agar was added to rest of the diet and mixed well again in the blender. The diet was poured into Petri plates and was allowed to cool in a refrigerator. Single, pre-starved (3-4 hours), weighed (5-10 mg) five day old *S. litura* larvae were placed in
plastic culture bottles (6 cm high and 4 cm in diameter) containing pieces of treated diet/control. A bottle with a larva, in ten replicates, was kept for a single concentration. Six concentrations of each derivative were used in the bioassay. Treated diet was replenished every day till pupation. Observations on larval weight (after 3 & 7 days), pupal weight, deformed pupae, larval-pupal and pupal-adult intermediates, per cent adult emergence and deformed adults were recorded.

The analyses of GI$_{50}$ were carried out by using software package (Indostat services Hyderabad). From the data collected from insect growth regulatory activity, per cent reduction in larval/pupal weight was calculated as follows.

$$\frac{\text{Weight gain in control} - \text{Weight gain in treatment}}{\text{Weight gain in control}} \times 100$$

Different parameters such as larval mortality, larval–pupal intermediates, pupal mortality, pupal–adult intermediates, abnormal adults and normal adults were taken into consideration to assess the IGR effects. Larval and pupal weight reductions over control were also calculated. Larvae were weighed after 3 and 7 days of treatment. Dead larvae, dead pupae, and abnormal individuals were identified and compared with normal ones.

- **Dead larvae:** After treatment when the larvae were unable to move even after pressing with a brush they were counted as dead. Some of them were found unable to moult and died during the moulting process, while some were not able to grow and remained smaller.

- **Larval – pupal intermediates:** Larval – pupal intermediates retained larval characters and acquired partial pupal cuticle on head, thorax or abdomen. Pupal cuticle exhibited different degree of chitinisation.

- **Dead pupae:** Most of the dead pupae were darker in colour and smaller in size. Treatment with some of the compounds gave rise to abnormal pupae which were deformed or with incomplete chitinisation. These abnormal pupae could not emerge into adults.

- **Pupal – adult intermediates:** Insects were considered as pupal – adult intermediates when some adult characters were visible in the anterior portion such as antenna, eyes, legs, and forewings, whereas, the posterior part still
retained pupal characters. Some of the adults emerged were unable to cast their pupal exuviae completely.

- **Abnormal adults:** Abnormal adults were with shrunken and partially stretched/wrinkled wings. These adults were unable to fly and died after some time.

### 2.9.2 Study of Insect Growth Regulator Activity on *Dysdercus koenigii* 11-14

#### 2.9.2.1 Insect Bioassay of *Dysdercus koenigii*

**Culture:**

Eggs and adults of *Dysdercus koenigii* were obtained from fields of Jalgaon region. The insects were reared in the laboratory in glass jars containing moist soil. They were fed on cotton seeds, soaked overnight in water. The cultures were maintained at room temperature (27±2°C). Nymphs of different stages (instars) and adults were separated in different glass jars as soon as they emerged. Their age was counted from the day of their emergence.

**Treatment:**

The test compounds were dissolved in acetone (1 mg/ml). The required volume of the test solutions were then topically applied with the help of microlitre syringe to the dorsal abdominal region of same aged 5th instar nymphs. The concentrations of the compounds used were 10, 15, 20, 30, 40 and 50 µg/nymph. The treated nymphs were placed back in the jars after the acetone had evaporated. At least three replicates (10 insects per replicate) were used for each dose of a compound. A parallel control group of nymphs treated only with acetone was set up with each experiment. The bioactivities of test compounds were determined by its effects on mortality (toxic/insecticidal effect), moulting and growth (growth inhibiting/regulating activity). 15-18

**Calculations:**

The results have been derived on percentage basis from the total number of 30 nymphs in three replicates. Based on the observed data, LD$_{50}$ values (lethal dose µg/nymph) were calculated using statistical computer program (Indostat Services, Hyderabad).

### 2.9.3 Antifungal Susceptibility Testing by Microbroth Dilution Method 15-21

The purified final compounds were evaluated for antifungal susceptibility testing by Microbroth Dilution Method according to the recommendations of the National Committee for Clinical Laboratory standards (NCCLS). The cultures used
were phytopathogens – *Magnaporthe grisea, Fusarium oxysporum, Dreschlera oryzae*, food spoilage yeasts - *Debaromyces hansenii, Pichia membranifaciens*, human pathogens - *Candida albicans* NCIM 3557, *Cryptococcus neoformans*. *C. albicans* and *C. neoformans* were isolates from clinical samples whereas other strains and *C. glabrata* were procured from National Centre for Industrially Important Microorganisms (NCIM), Pune, India. Appropriate amount of compounds were dissolved in dimethyl sulfoxide to get 100X final strength. The stock is then diluted 1:50 in RPMI-1640 medium and 200 µl from this is added to the first row of a 96-well microtiter plate. The compound were serially diluted two fold in successive wells to get a range of 4-512 µg/ml. Fungal yeast cells (~2x10⁴ cfu/mL, spores for phytopathogens), freshly grown in YPG broth in logarithmic phase, were suspended in the medium and inoculated (100 µl) in the wells of the plate. The microtiter plate was incubated for 48 h, and the absorbance was measured at 600 nm by using microtiter plate reader to assess cell growth. The MIC was defined as the lowest concentration exhibiting >90% inhibition of visible growth compared to growth of the control.

2.9.4 Cellular Toxicity Assay

The toxicity of Benzoyl Carvacryl Thiourea [BCTU (4 a-f)] and Benzoyl Carvacryl Urea [BCU (5 a-f)] derivatives was test by the red blood cell (RBC) lysis assay. The concentrations tested were in the range of 4-1000 µg/ml. The 2x desired concentration of the compound (in 750 µl of PBS) was mixed with 750 µl of 4% sheep RBC suspension in Eppendorf tubes and incubated at 37°C for 2 h. Triton X-100 (0.1% (v/v) in PBS was used as a positive control whereas 1% DMSO and PBS were used as negative controls. Tubes were centrifuged at 2,000 rev min⁻¹ for 10 min and the absorbances of supernatant were read at 540 nm. Percent haemolysis was calculated as: \[\frac{(A-B)}{(C-B)}\times100\], where A and B are the absorbance values of supernatant from the test sample and PBS (solvent control), respectively, and C is the absorbance value of supernatant from the sample after 100% lysis. The concentration (mean) causing 50% lysis (HC₅₀) was obtained from two independent experiments performed in triplicate.

2.10 REFERENCES


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