THEORETICAL ANALYSIS
### Chapter-3. Theoretical Analysis

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3.1 Theoretical analysis of antimicrobial activity

Media and chemicals

Nutrient Broth, Nutrient agar and 5mm diameter antibiotic assay disc were obtained from Hi-Media Laboratories Limited, India. Barium chloride dehydrate GR, concentrated sulphuric acid GR, Dimethyl sulphoxide GR, Sodium chloride AR and potassium dichromate were obtained from Ranbaxy Laboratories Ltd, Chemical Division, India. The synthesized compounds were assayed for antimicrobial activity against four registered bacterial isolates, which were obtained from National Center Cell Science (ACSS), Pune, India. The bacterial included two Gram positive bacterial isolates - *Staphylococcus aureus* NCCS 2079 and *Bacillus cereus* NCCS 2106 and two Gram negative bacterial isolates - *Escherichia coli* NCCS 2065 and *Pseudomonas aeruginosa* NCCS 2200. The bacteria were grown and maintained on nutrient agar (Hi-Media, Mumbai) and were subculture when needed.

Glass Wares and Apparatus

Glass Petridis, Glass tubes, Beakers, Erlenmeyer flask, Bacterial loop and measuring cylinder. All the glassware’s were Borosilicate grade. Digital electronics balance (Shankar scientific supplies, India), Yorco horizontal Laminar air flow bench (Yorco sales Pvt Ltd, New Delhi, India), Ausco incubator, Zone reader (Cintex industrial corporation, India), hot air oven, autoclave and UV/Visible spectrophotometer (Shimadzu corporation, Japan).
Antibacterial activity

The antibacterial activity of synthesized compounds was studied by the disc diffusion method\(^1\) against the following pathogenic organisms. The gram-positive bacterial screened were *Staphylococcus aureus* NCCS 2079 and *Bacillus cereus* NCCS 2106. The gram negative bacterial screened were *Escherichia coli* NCCS 2065 and *Pseudomonas aeruginosa* NCCS 2200.

The synthesized compounds were used at the concentration of 250 µg/ml using DMSO as a solvent. The amoxicillin 50 µg/ml was used as standard. (HI - media laboratories limited, Mumbai).

**Disc diffusion Method**

A Suspension of *Staphylococcus aureus* was added to sterile nutrient ager at 45\(^\circ\)C. The mixture was transferred to sterile petridishes to give a depth of 3 to 4 mm and allowed to solidify. Precautions were observed to produce uniform layer of medium on the plate. Sterile discs 5mm in diameter (made form what Mann Filter paper) were impressed in the solutions of synthesized compounds (250µg/ml) and maintain an untreated control sample for comparison. Leave the plates to stand for 1hr at room temperature as a period of preincubation diffusion to minimize the effects of variations in different time. Then the plates were incubated at 37 \(^\circ\)C for 24 h and observe for antibacterial activity. The diameter of the Zone of inhibition was measured for each plate in which the zone of inhibition was observed. The average zone of inhibition was calculated and compared with that standard.
A similar procedure was adopted for studying the antibacterial activity against the other organisms.

**Antifungal activity**

The antifungal activity of synthesize compounds were studied by disc diffusion method against the organisms of *Aspergillus Niger* NCCS 1196 and *Candida albicans* NCCS 3471 / *Helminthosporium Oryzae*. Compounds were treated at the concentrations of 250 µg/ml using DMSO as a solvent. The standard used was Ketoconazole / Griseofulvin 50 µg/ml against both the organisms.

**Disc Diffusion Method**

A suspension of *Aspergillus Niger* NCCS 1196 was added to sterile sabouraud dextrose agar at 45°C. The mixture was transferred to sterile petridishes and allows solidifying. Sterile discs 5 mm in diameter (made from Whitman Filter paper) impressed in the solutions of synthesized compounds and control were placed on the surface of agar medium with forceps and pressed gently to ensure even contact. Leave the plates to stand for 1 h at room temperature as a period of preincubation diffusion to minimize the effects of variation in time between the applications of the different solutions. Then the plate were incubated at 37 °C for 48 h and observed for antibacterial activity. The diameter of the zone of inhibition was measured for the plates in which the zone of inhibition was observed. The average zone of inhibition was calculated and compared with that of standard. A similar procedure was carried out for studying the antifungal activity against the other organisms (Candida albicans).
3.2 Theoretical analysis of polarographic studies

Preparation of the solutions of the compounds investigated and Other reagents

The chemicals employed in the studies are generally of analytical reagent grade. But whenever it is necessary, the chemicals are purified by the standard procedures reported in the literature. In case of solid substances, known amounts of compounds are weighed into standard flask and dissolved in distilled water. In the case of liquids, requisite volume of the liquid is placed with the help of a burette into the standard flask, dissolved in distilled water and the resulting solution is made up to the mark. The solutions thus prepared are standardized by standard procedures if necessary.

Buffer solutions

Britton-Robinson buffer solutions [1] are prepared from the stock solutions of boric acid, phosphoric acid, acetic acid and sodium hydroxide. The pH is checked with ELICO pH meter, model LI – 10, M/s. ELICO Private Limited, Hyderabad, India.

Mercury

Analar mercury is further purified chemically according to the procedure described by Vogel and is vacuum distilled.

Description of the instruments used

1. pH meter

The pH measurements are made with pH meter model LI – 10 manufactured by M/s. ELICO Private Limited, Hyderabad, India. The instrument operates on AC (220 volts) mains and is provided with two
scales, one for 0.0 to 7.0 pH and the other for 7.0 to 14.0. Provision is made for temperature control in the instrument. Glass electrode EH–60 (Beckman type) and a saturated calomel reference electrode are used as indicator electrode and reference electrode respectively. Buffer solution of pH 4.0, for acid range and pH 9.0 for alkaline range is used respectively to calibrate the instrument.

2. Recording Polarograph

A CL–25 Pen Recording Polarograph manufactured by M/s. ELICO Private Limited, Hyderabad, India is used to record current voltage curves. It consists of three units, (A) Dropping mercury electrode, (B) Mains operated Polarograph unit and (C) Mains operated self balancing strip chart recorder. The required span EMF can be adjusted with range selector switch. The electrolysis current is made to pass through a precision resistor placed in series with the cell, and millivolt drop (IR) is fed to a self balancing potentiometric strip chart recorder, and is recorded on the X-axis. The required current sensitivity is obtained with the help of (1) current sensitivity and (2) recorder sensitivity knob. The movement of chart paper is synchronised with a span drive motor and the span scanning voltage is represented on the Y–axis.

3. Polarographic cell

The polarographic cell used was a double walled beaker made up of corning glass fitted with a rubber cork. Dropping mercury electrode was inserted through one of the holes through which the
mercury drops. Calomel electrode was used as the reference electrode and this was connected to the cell through a salt bridge.

Nitrogen gas from the cylinder was padded through alkaline pyrogallic acid solution to remove any trace of oxygen present and through the supporting electrolyte solution and was allowed to bubble through the electrolyte solution present in the cell. This was done to remove the dissolved oxygen from the electrolytic solution which otherwise gives polarographic waves for the reduction of oxygen.

The air was allowed to leave the cell through an outlet fitted with water, preventing air re-entering the cell again.

Water was allowed to circulate at constant temperature from a thermostat between the inner and outer walls of the double walled cell.

The capillary having the characteristics $1.80 \text{ mg}^{2/3} \text{ s}^{-1/2}$ at $h = 80 \text{ cms}$ is employed in the studies.

4. **Cyclic voltammeter**

The cyclic voltammeter used consists of an X–Y recorder (Model RE 0074), a PAR 175 Potentiostat and an PAR 175 Universal Programmer. A single compartment cell model 303 SMDE supplied by PAR with silver wire as reference electrode and platinum wire as counter electrode is used in the studies. A stationary mercury drop electrode (SMDE 303) with a drop area $0.0096 \text{ cm}^2$ is used as the working electrode.
In some studies BAS (Bioanalytical System) 100 A electrochemical analyser supplied by USA is used instead of the equipment described earlier. A single compartment Metrohm cell with calomel electrode and a platinum foil is used in the studies. A hanging mercury electrode (HMDE 303 model) with a drop area of 0.024 cm\(^2\) is used as the working cathode.

The circuit diagram for cyclic voltammetric experiment is shown schematically in Fig. VI.1.1 which depicts

(a) Function generator

(b) Potentiostat

(c) Recorder

(a) **Function generator**

This generates the desired triangular potential wave which is applied to the electrochemical cell through Potentiostat.

(b) **Potentiostat**

The conventional three-electrode Potentiostat is connected to the working, reference, and auxiliary electrodes immersed in the test solution placed in the cell. It controls the potential of the WE with respect to the RE while simultaneously measuring the current flowing between the WE and the AE. The Potentiostat performs three functions:

(a) It controls the applied potential, which is potential difference between the WE and RE (the applied potential controls what half reactions occur at the WE)
(b) It allows to pass current between the WE and AE without passing current through the RE (which would change its potential if current did pass through it) and

(c) It converts the cell current to a voltage for recording devices.

A Potentiostat must be able to bring the potential of the WE (with respect to the RE) to the desired level in a short enough time. The time taken by the Potentiostat for controlling the WE potential is called the rise time. The potentiostat’s internal feedback circuits prevent all but a very small current from flowing between the WE and RE. Because the very basis of voltammetry is the control of electrode potential, a function generator is required to provide the potential sweep or pulse sequence to be applied to the WE. Most modern Potentiostat include a built-in sweep and / or pulse generator, and those which are interfaced to a computer usually rely on the computer to generate the desired waveform. The inputs to the potentiostat are the connections to the electrodes in the cell. The outputs from the potentiostat are signal lines reflecting the current and potential of the WE(s).

(C) Recorder

X – Y recorder is a plotter which simultaneously plots current versus voltage.

Technique of cyclic voltammetry

This technique of cyclic voltammetry consists of applying voltage sweep to the working electrode to generate triangular wave with
reference to the reference electrode of the cell, simultaneously monitoring the resulting current between the working electrode and the inert electrode. The current converted voltage when fed to X – Y recorder results in the observation of a cyclic voltammogram.

**Potentio scan**

Controlled potential electrolysis at mercury pool cathode was carried out at constant potential using Potentiostan PAR 173. The potential corresponding to limiting current of polarographic wave of the said compound is set on the potentiostan by setting the initial potential knob in clockwise direction, keeping the operating selector is turned to the position I, stand potential flows through the cell at the desired constant potential set earlier which can be read from the meter. As the electrolysis proceeds, the fall in the current is followed as a function of time.

**5. Thermostat**

A circulating type thermostat, supply by M/s. Thoshniwal, Bombay, India, with water as the thermostatic bath liquid was employed to maintain a constant temperature with the range of ± 0.01°C.

**6. Spectrophotometer**

A Synstronics visible spectrophotometer model 106 manufactured by M/s. Synstronics Instrument, Ahmedabad, India is used in the studies. The instrument is a single beam spectrophotometer having a granting of 600 lines/mm and the wavelength range is from 340 nm to 960 nm. The nominal spectral
width is 20 nm, which is constant over the entire range. Full scale deflection is obtained over the wavelength range 340 – 600 nm. By adding a red filter and by interchanging the phototube the range is extended to 960 nm. The light source in the instrument is a 15 watt tungsten iodide filament lamp operated by an AC regulated power supply. High regulation in the power supply enables the lamp to function as a constant light intensity source. Matched glass cuvettes of 1 cm path length are used in the studies. The wavelength accuracy is ± 5 nm. The optical density values are correct upto the third decimal place, ± 0.001 are obtainable through the 4 digital display with the instrument.

7. Electrolysis Cell

In its simplest form, the electrolysis cell is a single piece of glassware capable of holding an appropriate volume of a test solution containing one or more electroactive analytes. The cell is then maintained oxygen free by passing nitrogen over the solution through nitrogen inlet. The electrochemical cell consists of three electrodes which are immersed in this solution and are electrically connected to the potentiostat. The Reference electrode used is SCE, which is often isolated from the solution by a salt bridge to prevent contamination by leakage from the RE. The Auxiliary electrode (platinum foil) and the working electrode hanging mercury drop electrode (HMDE), is placed directly into the solution. Custom glassware designs include convenient fittings for mounting electrodes, gas inlets and outlets for purging oxygen and temperature jackets. Since the limiting (peak)
current in any type of voltammetry is temperature dependent, the cell is thermo stated for the required temperature.

8. Electrodes

In the present work three electrode system is used i.e. WE / AE / REs. The Reference electrode used is SCE which is often isolated from the solution by a salt bridge to prevent contamination by leakage from the Reference electrode. The platinum foil is used as Auxiliary electrode and the working electrode is HMDE.

**General polarographic procedures**

**(A) Polarographic and cyclicvoltammetric procedures**

Polarographic behaviour of the title compounds at different

(i) pH values

(ii) Concentrations of the substrate,

(iii) Heights of the mercury column, is studied adopting the following procedures.

**(i) Effect of pH**

8.0 ml of the buffer solution of desired pH (1.1 – 10.1), 2 ml of the stock solution of the substrate (1.0 x 10^-2 M) in dimethylformamide (DMF), 6 ml of dimethylformamide (DMF) and 4.0 ml of distilled water are mixed thoroughly in the polarographic cell and the polarograms are recorded after removing the dissolved oxygen by passing pure and dry nitrogen gas through the solution for about fifteen minutes.
(ii) **Effect of concentration**

8.0 ml of required buffer solution (pH 4.1) and a known aliquot of the substrate solution (1.0 x 10^{-2} M) and the requisite volume of dimethylformamide are mixed in the polarographic cell to make the total volume 20 ml and the polarogram is recorded after deprecation.
iii) Effect of mercury column height

8.0 ml of the buffer solution (pH 4.1 and 8.1), 2.0 ml of the substrate (1.0x10^-2 M) stock solution, 6.0 ml dimethylformamide (DMF) and 4.0 ml of distilled water are mixed in the polarographic cell and the polarogram is recorded at different heights (80, 70, 60, 50 and 40 cms) of mercury column.
3.3 Theoretical analysis of cyclic voltammetric studies.

Cyclic voltammetry is yet another important voltammetric technique which provides the means to examine the nature or pathway of an electrochemical reaction in detail. The current potential curves at stationary electrodes (like platinum electrode, hanging mercury drop electrode – HMDE, glassy carbon electrode) in unstirred solutions depend on the rate of change of applied potential. At a constant potential, the reduction or oxidation of an electroactive substance depletes a layer of solution that extends further from the electrode surface into the bulk of the solution as time proceeds, resulting in a current decrease.

Consider the electrode reaction of the type,

\[ \text{O} + \text{n}e^- \rightarrow \text{R} \]  

At positive potentials the rate of reduction is negligible, the current due to the reaction will be very small. When the potential is scanned in the cathodic direction, the principal effect is the increase in current resulting from an increase in the ratio of the concentrations \( \frac{C_{\text{O}}}{C_{\text{R}}} \) as per the Nernst equation for a reversible reaction. If the reaction of the O – R couple is irreversible, the increase in current results from the increased rate of reduction of O.

As the applied potential moves to negative values, the ions or molecules of the oxidised species (O) are reduced as rapidly as they arrive at the electrode surface. This situation is encountered at potentials corresponding to the limiting region of the DC polarograms. At these potentials the two opposing effects discussed cause the
current first to pass through a maximum value called the peak current, \( i_p \), and then decrease again. The shape of peak polarogram for linear sweep voltammetry can be visualized at the superposition of an \( i-t \) decay curve on a steady state polarogram. The rate of reduction is faster compared to the rate of arrival of O at the electrode surface. However, it is similar to what is existing in the current decrease at higher negative potentials.

Voltammetry with linear potential sweep can be used as a single or multi-sweep technique. Voltammetry with stationary electrodes has several advantages over conventional polarography such as

(a) a wide range of potential sweep rates can be used

(b) the experiment can be completed in a few seconds as against 5 to 10 minutes required in polarography and

(c) The method is more sensitive than conventional polarography with DME.

However, there is a disadvantage in the analysis of mixtures, this technique is difficult because of the uncertainties in the baseline of the second process\(^{226}\).

Fig. IX.1.1 represents a typical cyclic voltammogram for the reversible reduction reaction

\[
O + \hat{e} \rightleftharpoons R \quad (2)
\]

Cyclic voltammetry is a multi-sweep technique in which the potential is varied in the form of an isosceles triangle. At \( t = 0 \), the potential sweep begins at zero volt. The potential is swept at a
constant rate (dE/dt = V) until the switching potential \( E_s \) is reached. At this point the direction is reversed and the potential is swept back. The potential sweep may be continued for as many cycles as desired.

In the polarographic experiments, the changes in potentials are such that the rate of diffusion of oxidised and reduced forms to and from the electrode surface keep the system in equilibrium with the bulk solution at all times. In cyclic voltammetric experiments, the rate of variation of potential is too rapid for diffusional processes to maintain equilibrium with the bulk of the solution.

The relations in peak voltammetry for reversible, quasi-reversible and irreversible systems were considered by Matsuda and Ayabe\textsuperscript{227} for linear diffusion to a plane electrode area. At square centimeter, the peak current obtained for the forward polarisation is described by the Randles Sevcik equation,

\[
i_p = 2.687 \times 10^5 A D^{1/2} C^{1/2} V^{1/2} \tag{3}
\]

where

- \( i_p \) is the peak current in micro amperes
- \( C_0 \) is the bulk concentration of oxidant in millimoles litre\(^{-1}\)
- \( V \) is the scan rate of volts S\(^{-1}\).
- \( A \) is the area of the electrode in cm\(^2\), and
- \( n \) is the number of electrons transferred

The numerical constant used in equation above was given by Nicholson and Shain\textsuperscript{228}.

The equations for reversible reactions were worked out by Shain and Nicholson\textsuperscript{228-230}. The peak potential, \( E_p \) is given by the equation,
EP = E1/2 - 0.029/n  \hspace{0.5cm} (4)

Where E_{1/2} is the polarographic half-wave potential in volts. The potential of the anodic peak on a voltammogram of the reduced species is 0.029/n volts more positive than the E_{1/2}.

For the reversible reduction of O to R, the half-peak potential, \( E_{P/2} \) is given by

\[ E_{P/2} = E_{1/2} + 0.028/n \]  \hspace{0.5cm} (5)

Conversely, the anodic half-peak potential is 0.028/n volts more negative than the half-wave potential. The fundamental criterion of reversibility is therefore,

\[ (E_{P/2})_c - (E_{P/2})_a = 0.056/n \text{ V} \]  \hspace{0.5cm} (6)

The peak current \( i_p \), for a total irreversible reduction of O to R is given by

\[ i_p = 2.985 \times 10^5 \times n \ (\alpha_{na})^{1/2} \ A \ D_0 V^{1/2} \ C_0 V^{1/3} \]  \hspace{0.5cm} (7)

The numerical constant is determined by Nicholson and Shain^3. The peak potential for an irreversible reaction is given by the expression,

\[ E_p = E_0 - 0.059/\alpha_{na} [0.4565] + \log \alpha_{na} D_0 V^{1/2} / K_{s,h} \]  \hspace{0.5cm} (8)

While half peak potential, \( E_{P/2} \) is described by

\[ E_{P/2} = E_p + 0.048/\alpha_{na} \]  \hspace{0.5cm} (9)

In equation (7) and (8), \( \alpha \) is transfer coefficient, \( n_a \) is the number of electrons transferred in the slow step and \( K_{s,h} \) is the standard rate constant.

Since fast sweeps are employed, multi-electron transfer processes, fast enough to appear reversible in DC polarography may
appear to be irreversible in cyclic voltammetry. As the sweep rate is increased, the slowness of the electron transfer step becomes more and more pronounced and deviations from Nicholson behaviour and become more marked. On the other hand, the fast electron-transfer step followed by a slow chemical transformation of the product into an electroactive species appear to be irreversible in ordinary polarography because the stable form of the product does not give an anodic wave having the same half wave potential as the cathodic wave of the starting material. If the sweep rate is high, such a process may appear to behave reversible because the inactivation can then occur only to limited extent and the deviations from reversible behaviour decrease as the sweep rate increases to a point where the finite speed of electron transfer becomes perceptible.

Nicholson\textsuperscript{5} has used the separation in anodic and cathodic potentials for the evaluation of the rate constants of electron transfer. The cyclic voltammetry of a number of organic compounds with a view to study the intermediate stages of electrochemical reactions has been reported by Adams\textsuperscript{231-233}. Nicholson and Shain\textsuperscript{229} made use of the cyclic voltammetric technique for the study of ECE mechanism operative in organic electrode as detailed in the scheme 5.8.1.

In cyclic voltammetric study the reaction responsible for the manifestation of the cathodic peaks may be charge transfer (reversible or irreversible) reaction or charge transfer reaction coupled with a chemical reaction. This chemical reaction may proceed the charge
transfer or succeed the charge transfer reaction. Further the reaction can be an irreversible.

1. Reversible charge transfer

   \[ \text{O} + \text{ne} \rightleftharpoons \text{R} \quad (10) \]

2. Totally irreversible charge transfer

   \[ \text{O} + \text{ne} \xrightarrow{k} \text{R} \quad (11) \]

3. Chemical reaction preceding a reversible charge transfer

   \[ \text{Z} \xrightarrow{k_f} \text{O} \xleftarrow{k_b} \quad (12) \]

   \[ \text{O} + \text{ne} \rightarrow \text{R} \quad (13) \]

4. Chemical reaction preceding an irreversible charge transfer

   \[ \text{Z} \rightarrow \text{O} \quad (14) \]

   \[ \text{O} + \text{ne} \xrightarrow{K} \text{R} \quad (15) \]

5. Reversible charge transfer followed by reversible chemical reaction

   \[ \text{O} + \text{ne} \rightleftharpoons \text{R} \quad (16) \]

   \[ \text{R} \xrightarrow{k_f} \text{Z} \xleftarrow{k_b} \quad (17) \]

6. Reversible charge transfer followed by irreversible chemical reaction

   \[ \text{O} + \text{ne} \rightleftharpoons \text{R} \quad (18) \]

   \[ \text{R} \xrightarrow{k} \text{Z} \quad (19) \]
7. Irreversible catalytic reaction following the reversible charge transfer

\[ \text{O} + \text{ne} \xrightarrow{k_1} \text{R} \] (20)

\[ \text{R} + z \xrightarrow{k_1} \text{O} \] (21)

8. Irreversible catalytic reaction following the irreversible charge transfer

\[ \text{O} + \text{ne} \xrightarrow{k} \text{R} \] (22)

\[ \text{R} + z \xrightarrow{k} \text{O} \] (23)

catalytic reaction. Thus eight categories of mechanisms are broadly envisaged by Nicholson and Shainas detailed explain the shape and characteristic of the cyclic voltammogram.

**Diagnostic criteria**

Even though many correlations which could be made generally, few only can be practically used with great ease to arrive at the type of the mechanism. Most widely used are the variation – with changes in rate of voltage scan of the cathodic peak potential, half-peak potential and the ratio of the anodic to the cathodic peak currents. These diagnostic relations are useful for qualitative characterisation of unknown systems since only trends in the experimental behaviour are required. The variation of current function \( \left( \frac{i_{pc}}{V^{1/2}} \right) \) or \( \frac{\Delta E_{p/2}}{\Delta \log V} \) with scan rate \( V \) can be conveniently used for the diagnosis. The graphs characteristics of these variations for the eight mechanisms listed earlier are presented in the chart. 5.8.1 and 5.8.2.