# CHAPTER 2
# MATERIALS AND METHODS

This chapter introduces the materials, preparative methods, characterization and corrosion monitoring techniques employed for the natural and synthetic corrosion inhibitors.

## Natural corrosion inhibitors

### *Preparation of extracts*

Ethanol extracts, prepared from plant leaves, were acted as inhibitor solutions against mild steel corrosion in acid media. Plant leaves were collected from different areas of Thrissur District. They were washed with distilled water to clean any dust particles attached to them and shade dried for two weeks. Dried leaves were then made fine grain using a grinder. 5 g of the powdered sample was refluxed with 100 ml ethanol for 4 hrs. It was then filtered after keeping overnight and concentrated into 50 ml. The selected plants and the major phytochemicals present in them, used for the application as corrosion inhibitors are listed in Fig. 2.1.

Experimental and quantum mechanical calculations were applied to evaluate the inhibition potential of plant leaves. Literature survey was the essential source for the identification of the principal constituents of the leaves. Among the various phytochemicals, one or two components were then selected to explain the mechanism of corrosion inhibition experimentally and theoretically.

### *Materials and medium*

Weight percentage of the mild steel used: carbon 2.95 % and iron 97.05%. Mild steel metal sheet was cut into 1x1x0.059 cm dimension, and then they were

smoothened using an abrasive material with different grades such as 100, 220, 440, 800, 1000, 1500 and 2000 and cleaned with distilled water, followed by acetone.

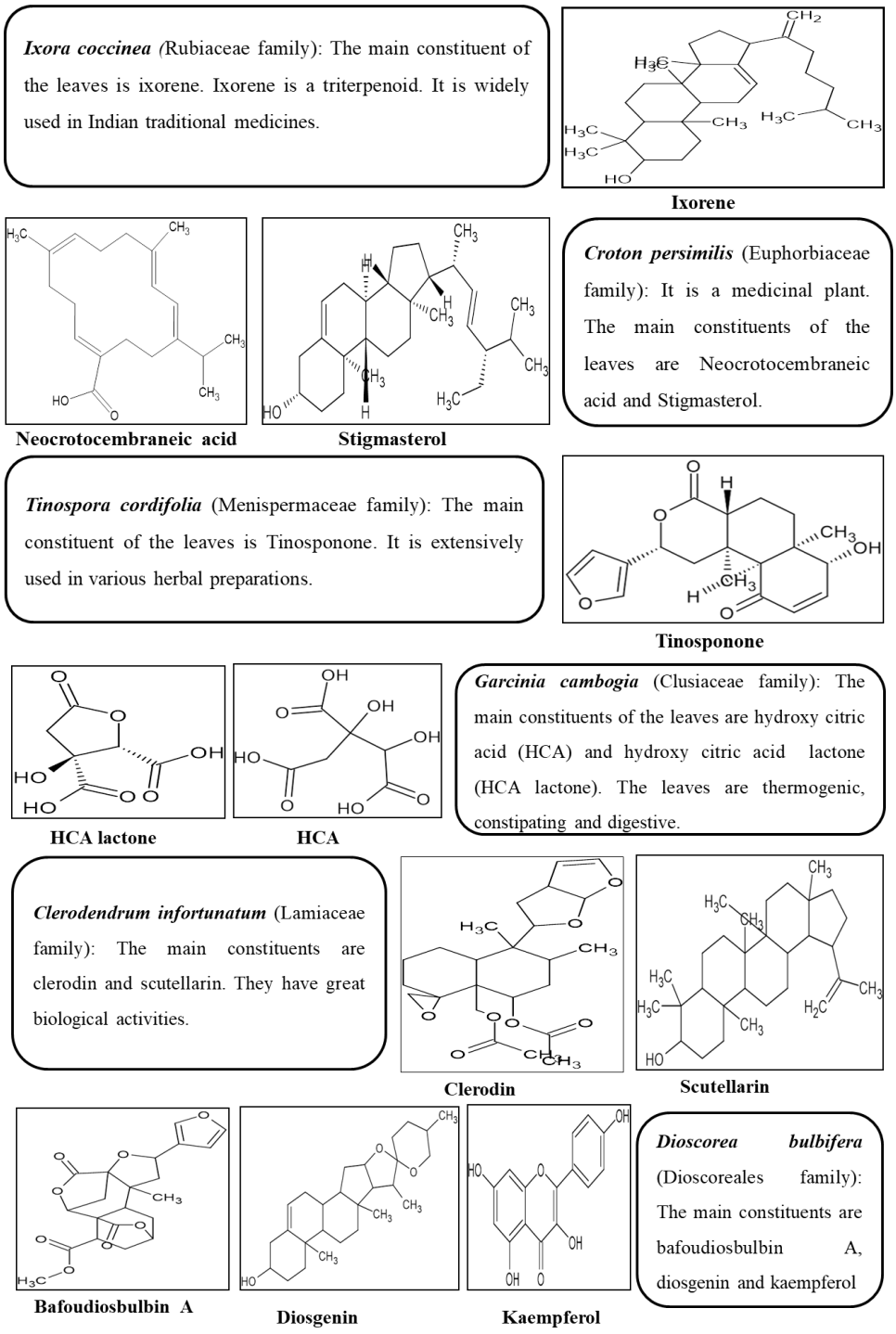



Fig. 2.1: Plant extracts and structure of significant constituents

Analytical pure (Merck) HCl and $H_2SO_4$ were used for preparing 1 M HCl and 0.5 M $H_2SO_4$. To analyze the effect of temperature on corrosion rate, a thermostat was used at temperatures 303 K, 313 K, 323 K and 333 K.

Ethanol, Mayer's reagent, chloroform, potassium ferrocyanide, NaOH, lead acetate, con. $H_2SO_4$ of Merck was used to conduct the phytochemical screening.

Merck samples of chromium acetate, cobalt acetate, copper acetate, manganous acetate, ferric chloride, sodium chloride and zinc acetate were used to determine the metal-binding capacity of alcoholic extract of plant leaves.

***Spectral analysis***

❖ ***FTIR spectroscopy***

IR spectra of extracts were examined to recognize the functional groups present in extracts for the studies and Spectrum Two FTIR spectrometer (Perkin Elmer, USA) was employed.

❖ ***UV-Visible spectroscopy***

The UV-Visible absorption spectra of 5 ppm plant extract, 5 ppm metal salts and an equal mixture of these two solutions were recorded using UV-Visible spectrophotometer, Shimadzu UV-1800, to study the metal-binding ability of plant extract. Chromium acetate, cobalt acetate, copper acetate, manganous acetate, ferric chloride, sodium chloride and zinc acetate were used to prepare 5 ppm metal salt solution.

***Screening of phytochemicals***

The phytochemicals of plant extract were tested by the standard methods[125,126,127,128]:

- Mayer's test: It is an identification test for alkaloids. 3 drops of freshly prepared Mayer's reagent were mixed with 1 ml of plant extract in a test tube. If it precipitates in white colour, the presence of alkaloids in the extract can be confirmed.

- Salkowaski's test: It is an identification test for steroidal compounds. 2 ml of chloroform was used to dissolve 5 ml of extract in a test tube and then was mixed with conc.$H_2SO_4$ to form a lower layer. Occurrence of reddish-brown colour at the interface is the confirmatory test for steroids in the extract.
- Test for phenolic compounds: 1% of ferric chloride solution was added to 1% potassium ferrocyanide in equal amounts. Then it was mixed with 2 ml of extract in a test tube. Visibility of bluish-green colour was accounted for the presence of phenolic compounds.
- Test for flavonoids: 3 ml of extract was mixed with 2 ml of dilute NaOH solution. Presence of yellow colour indicates the existence of flavonoids.
- Test for Terpenoids: 1 ml of extract was added with 0.5 ml of chloroform and a few drops of conc.$H_2SO_4$. A reddish brown precipitate points out the presence of terpenoids in the extract.
- Froth test: It is a positive test for saponins. About 0.5 g of extract was dissolved in 10 ml distilled water. The aqueous solution was closed tightly and shaken for 30 seconds gently. Then it was kept for 30 minutes. Presence of honeycomb froth above the surface was considered as the identification of saponins.
- Lead acetate test: It is an identification test for tannins. Freshly prepared 1% lead acetate was added to 5 ml of plant extract. Appearance of yellow precipitate was considered as a positive test for the presence of Tannins
- Tests for cardiac glycoside: Aqueous extract was mixed with 2 ml of conc. $H_2SO_4$. Development of reddish-brown colour was intended to the presence of steroidal aglycone part of the glycoside

- Tests for coumarin: 2 ml of extract was treated with few drops of alcoholic NaOH solution. Presence of coumarins can be verified by the formation of yellow colour.
- Test for quinones: 1 ml of extract was added to conc.$H_2SO_4$ solution. Appearance of the colour showed the presence of quinones.

***Weight loss measurements***

Weight loss studies were conducted with and without 1-5 v/v% concentrations of plant extract in both acid solutions for 24 hrs immersion period at different temperatures. Smoothened mild steel coupons were pre-measured, pre-weighed and immersed in 50 ml 1 M HCl and 0.5 M $H_2SO_4$ solutions separately by adding 0 - 5 v/v% concentrations of plant extract. After the completion of the period of immersion, metal coupons were weighed and calculated its weight loss. Using the resulted weight loss values, corrosion rate (υ) and inhibition efficiencies (η%) were estimated by equations (8) and (9), respectively.

***Electrochemical studies***

Electrochemical impedance spectroscopy (EIS), polarization studies and electrochemical noise studies were performed using Ivium CompactStat-e electrochemical work station and IviumSoft software with a three-electrode electrochemical cell. A pictorial representation of three-electrode assembly is shown in Fig. 2.2.

For all the electrochemical studies, a saturated calomel electrode behaved as the reference electrode. For EIS and linear polarization studies, a platinum electrode was acted as the counter electrode, and the mild steel having 1 $cm^2$ exposed surface area was employed as a working electrode.

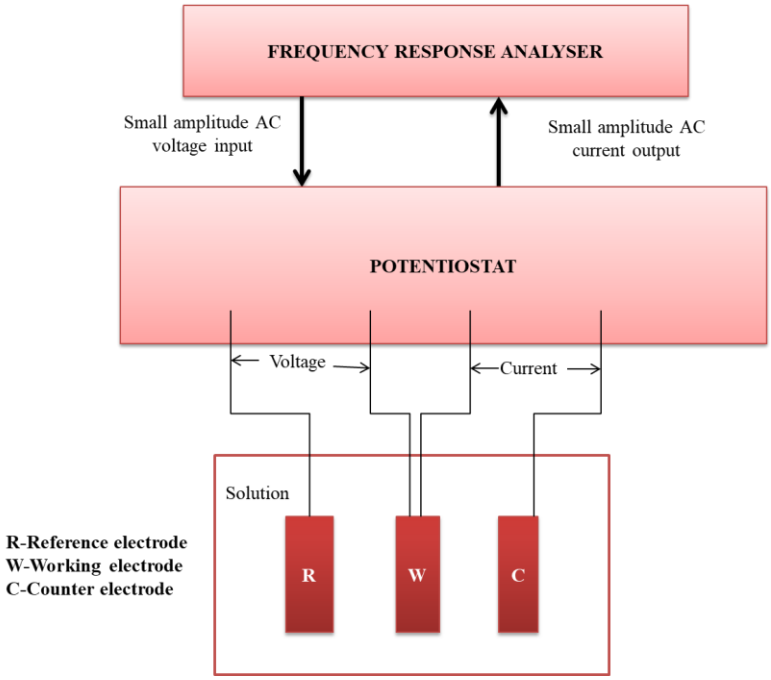



Fig. 2.2: Pictorial representation of a three-electrode assembly

The mild steel samples with 1 $cm^2$ exposed surface area were utilized as counter and working electrodes for electrochemical noise measurements. Reference electrode was same as EIS and potentiodynamic polarization studies. Measurements were carried at room temperature using 1 M HCl and 0.5 M $H_2SO_4$ with various plant extract concentrations (1- 5 v/v %).

The EIS parameters were measured over a frequency span of 1 KHz to 100 mHz and 10 mV signal breadth. Corrosion inhibition power of plant extracts was calculated from $R_{ct}$ values obtained from Nyquist plots by the equation (22).

Current-potential relationship of mild steel samples in the test solution was evaluated using the open circuit potential method by potentiodynamic polarization studies. Applied potential range for the working electrode was from -250 to +250 mV compared to the corrosion potential ($E_{corr}$) with a scan rate of 1 mV/sec. Surveillance of anodic and cathodic curves was accomplished to derive corrosion current density ($i_{corr}$), and then the percentage of inhibition potency ($\eta_{pol}$%) as shown in equation (30).

Electrochemical noise studies were carried out for a period of 1200s.

***Adsorption isotherms***

Mechanism of corrosion inhibition between inhibitor molecules and mild steel metal was illustrated using adsorption isotherms. Several adsorption isotherms like Langmuir, El-Awady, Frumkin, Temkin, Freundlich, and Flory-Huggins were considered for the study. Best suitable isotherm was detected using the correlation coefficient ($R^2$) value[44]. As the concentration of the inhibitor increases, the surface coverage ($\theta$) also increases. Adsorption of inhibitor molecules on the metal surface leads to decreased active surface area exposed to the acid media, thereby reducing metal corrosion.

***Scanning electron microscopy***

Surface characterization of mild steel coupons submerged in 1 M HCl and 0.5 M $H_2SO_4$ media comprising 5 v/v% of plant extract was examined by a scanning electron microscope JEOL Model JSM- 6390LV. The immersion period for the mild steel in contact with acid media and plant extract was 24 hrs.

***Atomic force microscopy***

Surface mechanism of plant extract on mild steel was further strengthened by an atomic force microscope WITEC ALPHA300 RA. Mild steel coupons employed for analysis and the immersion periods are the same as in SEM analysis.

***Quantum mechanical calculations***

Quantum mechanical parameters of active principles of plant extract were calculated using DFT method by GAMMES software. Correlation factor used was B3LYP and STO-3G as the basis set. This method provides a correlation between the corrosion inhibition efficiency of the inhibitor molecules and their electronic properties[129]. Calculated parameters such as $E_{HOMO}$, $E_{LUMO}$ and $\Delta E$ were used to evaluate the corrosion inhibition behaviour of the compound.

***Response surface methodology (RSM)***

Response surface methodology (RSM) is the established optimization technique in current years. It describes the effect of a quantitative response on process factors, individually or with one another, and generates an empirical model illustrating the proper quantity of processes[130]. Response is a dependent variable, and process factors are known as independent/predictor variables or factors. RSM can apply for maximizing or minimizing the responses.

Generally, response surfaces are performed by a second-order regression model. A second-order regression model is also called a full quadratic equation for 'k' number of factors, shown in equation (50).

$$Y = a_0 + a_1X_1 + \dots a_kX_k + a_{12}X_1X_2 + \dots a_{k-1,k}X_{k-1}X_k + a_{11}X_1^2 + \dots a_{kk}X_k^2 \quad (50)$$

The favoured method of response surface design is Central Composite Design, CCD[131]. Fig. 2.3 is a schematic representation of CCD that is a two-level full factorial or fractional factorial design having centre points and axial points. Centre point is positioned at the centre, whereas the axial points are located at the middle of the levels of a factor. So, the axial points are coordinated with (-1, 0), (1,0), (0, -1), and (0, 1). Fig. 2.3 is a $3^2$ factorial for which a full quadratic model can be suited for the response surface. When more centre points are inserted, the lack of fit has to be tested. The distance between the centre and the axial points is designated by α.

Accuracy of the prediction model can be evaluated by producing consistent and stable variance over the complete ranges of the independent/predictor variables. The statistical significance of an independent variable is verified by performing an analysis of variance (ANOVA). If the p-value is less than the significance level (0.05), then the full quadratic model of independent variables significantly influence the dependent variable[132]. When R-square and adjusted R-square values are close to unity, the model

parameters can perfectly explain the variation of dependent variables suggesting, a tremendous practical significance.

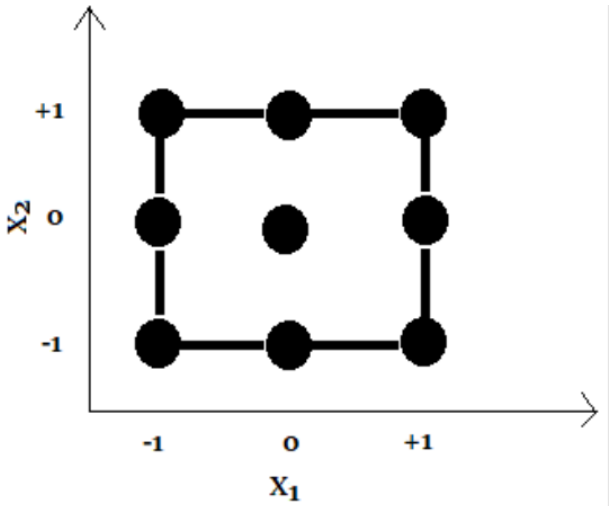



Fig. 2.3: Schematic representation of CCD with α=1

Normal probability plot gives information about the distribution of residuals[133]. If the residuals follow approximately a straight line, then it is a normal distribution for the residuals. The residuals vs fitted plots reveal the predictability of the residuals[134]. Lack of clear pattern in residuals vs fitted plot suggests no unpredictability of the residuals. Then, the residuals can be regarded as homogenous. Residuals vs the observation order plot imply the correlation between the observation number and the residuals.

Box-Behnken Design (BBD) is another attractive design for RSM to fit a second-order model[135]. BBD requires only three levels for each factor and a fewer number of experimental runs. BBD places a mid-level between the low-level and high-level factors, neglecting the extreme axial points as in CCD. Comparing to CCD, BBD uses face points beside the corner points. The assessment of the coefficients of a second-order model is more straightforward when a mid-level point is added. In BBD, $2^2$ full factorial is employed as the base design. Then, applying mid-levels for the other factors, orthogonal blocks are produced.

Analysis and demonstration for the results of BBD are the same as CCD. CCD is the traditional fractional factorial design of experiments. So, it has all the merits of fractional factorial design. In addition, CCD is rotatable, whereas BBD is nearly rotatable or rotatable for some particular designs[136]. CCD can make possible a fourth-order model since it contains five levels for each factor. At the same time, BBD can set only a second-order model because there are only three levels for each factor. BBD could be more beneficial for more well-informed processes in practice, while CCD could be more interested in comparatively unknown processes. That's why CCD is used frequently compared to BBD since most RSM studies are performed to find something new. Even so, for more refinement and optimization, BBD is the more precise method.

In short, both designs have their merits and demerits. The designers can select any of these two based on the optimization goals. Composite desirability function used for the optimization technique. Overall predictability for responses by the independent factors can measure in it.

❖ ***RSM for maximum corrosion inhibition efficiency***

RSM helps to discover the optimum parameter condition to attain the maximum inhibition efficiency. Experimental design and analysis were performed using Minitab 19 programming tool, and CCD/BBD was applied to include possible experimental runs. The independent factors used in CCD were temperature ($X_1$) and inhibitor concentration ($X_2$), whereas those in BBD were temperature ($X_1$), inhibitor concentration ($X_2$) and acid concentration ($X_3$). Inhibition efficiency was the corresponding response. Table 2.1 shows the level of independent factors of BBD/CCD with coded and uncoded forms. The generalized full quadratic model for the three factors $X_1$, $X_2$ and $X_3$ in BBD is shown in equation (51) and that in CCD is given in equation (52).

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (51)$$

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2 \quad (52)$$

where Y is the predicted value of corrosion inhibition efficiency. $a_0$ is a constant, $a_1$, $a_2$ and $a_3$ are constants indicating the effect of factors $X_1$, $X_2$ and $X_3$. $a_{12}$, $a_{13}$ and $a_{23}$ are interaction constants between the two factors $X_1X_2$, $X_1X_3$ and $X_2X_3$. Quadratic constants of $X_1$, $X_2$ and $X_3$ are represented by $a_{11}$, $a_{22}$ and $a_{33}$.

Table 2.1: Level of factors of BBD/CCD with coded and uncoded form

| Sl. No. | Factor | Code | Unit | Level of factors | | |
|---|---|---|---|---|---|---|
| | | | | Low (-1) | Center (0) | High (1) |
| 1 | Temperature | $X_1$ | K | 313 | 323 | 333 |
| 2 | Inhibitor concentration | $X_2$ | v/v% | 1 | 3 | 5 |
| 3 | Acid concentration | $X_3$ | M | 0.5 | 1 | 1.5 |

**Synthetic corrosion inhibitors**

***Materials and methods***

The Schiff base derivatives were synthesized from pyridine-2-carbaldehyde, pyridine-3-carbaldehyde and 2-acetylpyridine of analar grade chemicals procured from Fluka. The reagents hydroxylamine hydrochloride, semicarbazide hydrochloride, phenylhydrazine hydrochloride, sodium acetate, NaCl, KCl, $CaCl_2$. $2H_2O$, $MgSO_4$. $7H_2O$, $MgCl_2$. $6H_2O$, $NaHCO_3$ were acquired from E-Merck. The medium utilized for the synthetic purpose was pure ethanol.

❖ ***Preparation of metal samples***

For weight loss measurements, metal samples of 1x1x0.059 cm dimension were used and polished using emery papers. The exact surface area and thickness of the metal samples were accurately determined using vernier callipers and screw gauge, then weighed accurately using an electronic balance. For electrochemical studies, metal samples of 10x2x0.059 cm dimension were used and polished with emery papers.

❖ ***Synthesis of inhibitors***

1) Synthesis of N-hydroxy-1-(pyridin-2-yl)methanimine (NHP2M)

Ethanolic solution of pyridine-2-carbaldehyde (2 mmol) was carefully transferred to the mixture of an aqueous solution (5 ml) of hydroxylamine hydrochloride (2 mmol) and sodium acetate (4 mmol). It was refluxed on a water bath for 20 minutes, and then cooled in the ice bath. It yielded pale pink coloured crude- NHP2M, then filtered and dried.

2) Synthesis of N-hydroxy-1-(pyridin-3-yl)methanimine (NHP3M)

Ethanolic solution of pyridine-3-carbaldehyde was added to a 1:2 aqueous mixture of hydroxylamine hydrochloride and sodium acetate taken in a round bottom flask. After mixing thoroughly, it was refluxed on a water bath for 20 minutes. The clear solution was cooled in an ice bath. It was precipitated the white coloured compound - NHP3M. Then, it was filtered and dried.

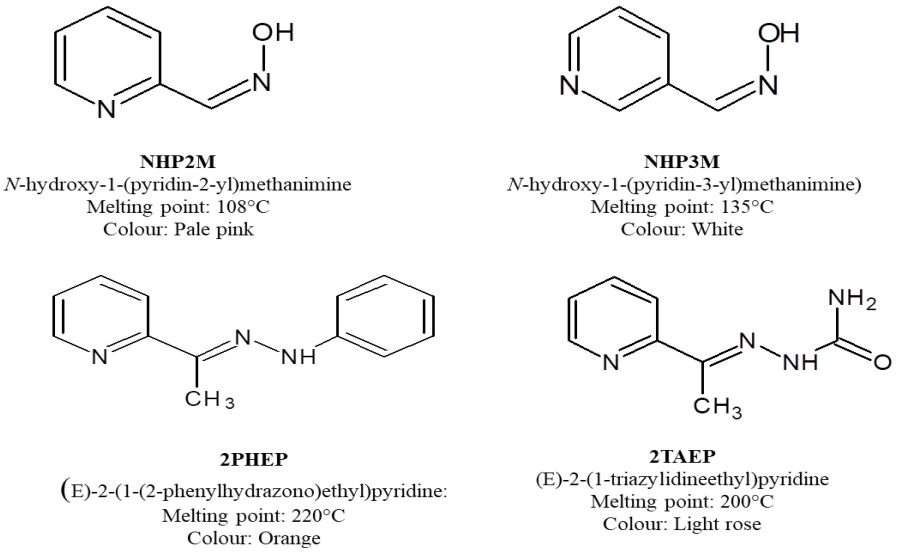
3) Synthesis of (E)-2-(1-(2-phenylhydrazono)ethyl)pyridine (2PHEP)

Ethanolic solution of phenylhydrazine hydrochloride mixed carefully with a hot ethanolic solution of 2-acetylpyridine. Refluxed for 3 hours on a water bath, then concentrated. It yielded orange crystals - 2PHEP.

4) Synthesis of (E)-2-(1-triazylidineethyl)pyridine (2TAEP)

A hot ethanolic solution of 2-acetylpyridine was transferred to an ethanolic solution of semicarbazide contained in a round bottom flask. Then the mixture was refluxed for 3 hours on a water bath. It concentrated and collected light rose coloured crystals - 2TAEP.

Fig. 2.4 shows the structures of the four synthetic inhibitors used in the present course of studies.




**NHP2M**
*N*-hydroxy-1-(pyridin-2-yl)methanimine
Melting point: 108°C
Colour: Pale pink

**NHP3M**
*N*-hydroxy-1-(pyridin-3-yl)methanimine)
Melting point: 135°C
Colour: White

**2PHEP**
(E)-2-(1-(2-phenylhydrazono)ethyl)pyridine:
Melting point: 220°C
Colour: Orange

**2TAEP**
(E)-2-(1-triazylidineethyl)pyridine
Melting point: 200°C
Colour: Light rose

Fig. 2.4: Structures of the four synthetic inhibitors understudy

❖ ***Preparation of artificial seawater broth***

An artificial salt mixture closely resembling the composition of ocean water for culturing marine bacteria was prepared.

Chemicals required:

NaCl - 24.600 g

KCl - 0.670 g

$CaCl_2$. $2H_2O$ - 1.360 g

$MgSO_4$. $7H_2O$ - 6.290 g

$MgCl_2$. $6H_2O$ - 4.660 g

$NaHCO_3$ - 0.180 g

Components of the artificial seawater media are separately weighed and suspended in 1 litre of double-distilled water. pH is read using a digital pH meter and adjusted to 7.5 using 1 N HCl or 1 N NaOH. The mixture is heated thoroughly to dissolve all components and allocated to 250 ml conical flasks. The resulting medium is sterilized by autoclaved at 15 lbs pressure at 121$^{o}$C for 15 min and allowed to cool to room temperature before inoculation.

❖ ***Culturing of marine bacteria***

Seawater was collected from Snehapuram beach, Vadanappilly, Thrissur. All samples were collected in the morning between 7.00 AM to 8.00 AM using a sterile glass bottle and transported immediately to the lab. 100 ml of this water were filtered through Whatman filter paper using Vacuum Filtration Apparatus. Microorganisms trapped in the filter paper is used as the inoculum for the culture. Sterilized and cooled artificial seawater media in the conical flask and is inoculated with a wire loop. Metal strip of dimension 1 cm x 1 cm was introduced into the flask and then incubated in a BOD incubator at 25$^{o}$C. Every day the flasks were shaken for one minute and examined for bacterial growth. The first subculture was done after 14 days of inoculation. Then the periodic subculturing was done on every seventh day. The metal strip was transferred to the new flask during the subculturing process.

***Isolation of corrosion causing bacteria***

To isolate the corrosion causing bacteria pour plate method[137] was used. Artificial seawater agar was made by incorporating 2% agar in the original seawater media. Microorganisms grown on artificial seawater media is serially diluted, and 0.1 ml from each dilution is transferred to a series of sterile Petri plates. After sterilization, the media is cooled to 45$^{o}$C and poured into the Petri plate. The media and inocula were mixed thoroughly by swirling and allowed to solidify. The plates were then incubated at room temperature for 2-3 days. Single-cell colony emerged was further isolated by streak plate technique using the same medium. Then it was subcultured on the artificial seawater agar slants and kept as stock cultures. Stock cultures for the experiments were preserved at 4$^{0}$C and subcultured frequently. Paraffin overlay technique was applied to keep permanent stock cultures[138]. Paraffin overlay technique employed an artificial seawater agar slant for inoculation of a single cell colony and incubated at room temperature (28 ±

2°C). Sterile liquid paraffin was used to envelop the top of the culture at the end of 18 hrs of incubation. At the same time, vials were overlaid by a sterile rubber cap.

***Identification of the bacterium***

Depends on the morphological and biochemical properties, the selected single cell colony MICBT7 was identified as summarized in Bergey's Manual of Systematic Bacteriology[139]. Further, to authenticate the identity, molecular ribotyping was also carried out. It was performed by employing the partial gene sequence of 16S rRNA.

❖ ***Isolation of genomic DNA***

It involved the following steps[140].

- Transferred mid-log phase culture of the bacteria (40 ml) into a sterile oakridge tube and centrifuged at 5000 rpm for 10 minutes at 4°C.
- Rejected the supernatant solution and dehydrated the pellet blot.
- Dissolved the cell pellet in 8.75 ml TE buffer.
- Then, 10 mg/ml Proteinase K and 10% SDS (l ml) were mixed thoroughly and incubated at 37°C for 1 hr.
- A 1:1 mixture of phenol and chloroform was transferred, mixed, and maintained for 10 minutes at 4°C.
- Centrifuged at 10,000 rpm for 10 minutes at $4^0$C, the supernatant was added to a sterile tube using a sterile cut tip.
- Above mentioned two steps were repeated three times.
- Aqueous phase was transferred into a sterile 50 ml beaker, with 0.1 ml of 5 M sodium acetate (pH 5.2) and 20 ml of isopropanol.
- DNA gets precipitated, collected and then washed with 70% ethanol.
- Dissolved the precipitated DNA in 1 ml TE buffer.

❖ ***Agarose gel electrophoresis***

Agarose gel electrophoresis[140] was performed to examine the nature of the DNA precipitated. In this electrophoresis, 0.8% (w/v) agarose gel was employed. After loading 10 µl of the DNA onto the agarose gel, electrophoresis was conducted at 80 V for 1 hr. In other words, up to the migrating dye (Bromophenol blue) had passed over a two-thirds distance of the gel, electrophoresis continued. Lambda DNA cut with EcoR I and HindIII (Bangalore Genei) was applied as the marker. 0.5 mg/ml ethidium bromide solution was employed to stain the agarose gel for 20 minutes. A UV transilluminator was operated to give the exact vision of the gel.

❖ ***Ribotyping***

Universal primer pair for 16S rDNA was applied for executing Ribotyping. A fragment of the 16S rRNA gene (l.5 kb) was amplified from the genomic DNA[141]. 16S rDNA was amplified by employing the forward (l6SF) and reverse (l6SR) primers with the corresponding sequence as follows:

Sequence:

16SF 5' AGTTTGATCCTGGCTCA 3'

16SR 5' ACGGCTACCTTGT TACGACTT 3'

❖ ***Polymerase Chain Reaction (PCR)***

A thermal cycler (Eppendorf master cycler personal) rendered PCR function under a set of conditions.

*PCR mix composition*

Deionized water - 12.5 µl

Enyme buffer - 2.5 µl

dNTP - 10 mM

Forward primer - 10 picomol

Reverse primer - 10 picomol

Sample DNA - 50 mg

*Taq* polymerase (1/10) - 0.6 U

*PCR conditions*

- Initial denaturation at 94°C for 2 minutes.
- Denaturation at 94°C for 45 seconds.
- Annealing at 59°C for l minute.
- Primer extension at 72°C for 2 minutes.
- Steps 2, 3 and 4 were repeated 29 times.
- Final extension at 72°C for 10 minutes.
- Hold at 4°C.

***Microbial corrosion monitoring methods***

The generalized initial procedure for weight loss and electrochemical studies can be explained using Fig. 2.5. The concentration of inhibitor was 100 ppm for all corrosion studies.

❖ ***Weight loss measurements***

In weight loss measurements, the metal samples of 1 $cm^2$ were immersed in artificial seawater broth in a hanging position with the help of fishing lines for 21 days in three systems: control (without bacteria), biotic (with bacteria) and biocide (with bacteria and inhibitor). Corrosion rate and inhibition efficiencies were calculated from the weight loss measurements data using the equations (8) and (9). The rate of corrosion was expressed in terms of mm/year.

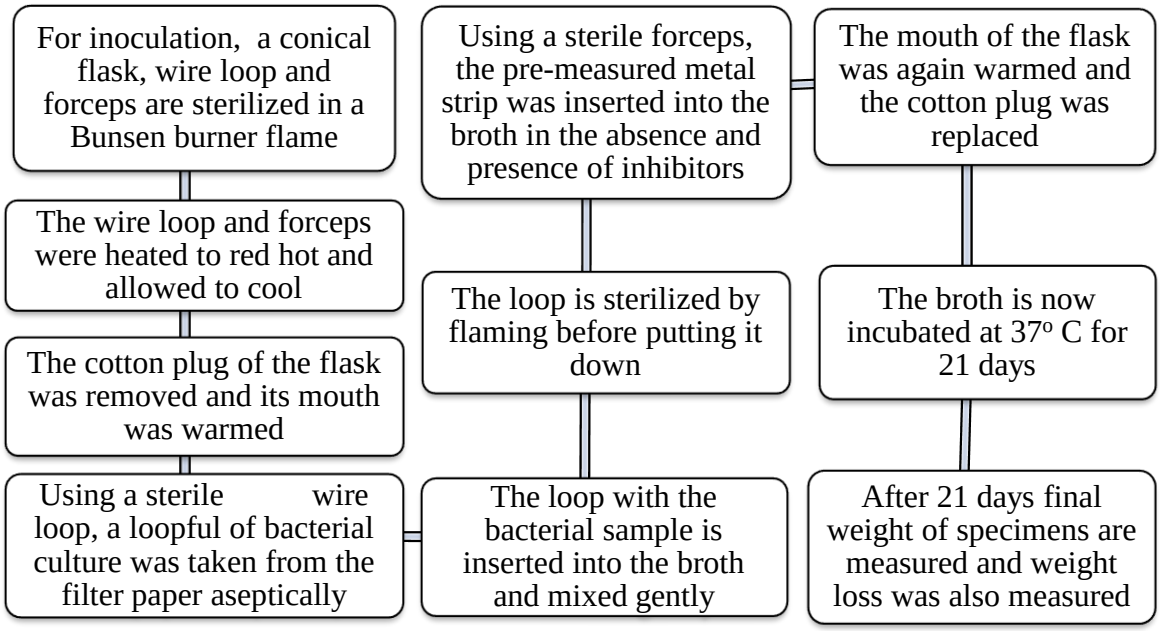



Fig. 2.5: Schematic representation of weight loss measurements

❖ ***Electrochemical impedance spectroscopy (EIS)***

Ivium compactstat-e electrochemical system was used for EIS studies. It was a three-electrode assembly. A saturated calomel electrode (SCE) was used as the reference electrode, and a platinum electrode with 1 $cm^2$ area was acted as the counter electrode. Working electrode was a metal sample with an exposed surface area of 1 $cm^2$ immersed in 100 ml artificial seawater broth containing control, biotic and biocide for 21 days (504 hrs) before the electrochemical measurements. EIS measurements were started after attaining a constant potential called OCP. It was carried out in the frequency range from 1 KHz to 100 mHz with an amplitude of 10 mV as an excitation signal. The percentage of inhibitions from impedance studies were estimated using charge transfer resistance values by the equation (22).

❖ ***Potentiodynamic polarization studies***

Electrochemical polarization studies of mild steel samples in control, biotic and biocide were conducted by recording anodic and cathodic potentiodynamic polarization curves. Polarization plots were derived in the electrode potential range from -250 to

+250 mV vs corrosion potential ($E_{corr}$) at 1 mV/sec scan rate. Corrosion current densities ($i_{corr}$) were rendered by extrapolating anodic and cathodic curves to the potential axis. The percentage of inhibition efficiency ($\eta_{pol}$%) was calculated from $i_{corr}$ values using the equation (30). Polarization resistance ($R_p$) values were acquired from the slope analysis, and inhibition efficiency was estimated using the equation (33).

***Surface analysis***

❖ ***X-ray diffraction spectroscopy (XRD)***

Mild steel surfaces immersed in control, biotic and inhibitor systems after 21 days of incubation were used for X-ray diffraction spectroscopy (XRD). A computer controlled XRD system (PANalytical) was performed among 5º and 90º - 2θ with copper K-alpha radiation at a 40 kV, 25 mA rating.

❖ ***FTIR spectroscopy***

After 21 days of incubation, the mild steel coupons were taken outside the artificial sea water medium, and the surface film was scraped using a sterile spatula. FTIR spectra of the scratched corrosion products on the mild steel were conducted for control, biotic and inhibitor systems using KBr pellet method. The spectrum was recorded in the range 400-4000 $cm^{-1}$ employing Shimadzu IR Affinity-1 model FT-IR spectrophotometer.

***Microscopic surface analysis***

After 21 days of incubation, the mild steel coupons were taken outside the artificial sea water medium and examined using a high-resolution optical microscope (Leica Stereo Microscope-No. S8ACO). This analysis focuses on studying the surface modifications that occurred for mild steel coupons when the metal coupons were exposed in control, biotic and inhibitor systems.

***UV-Visible spectroscopy***

UV-Visible spectra of Schiff base inhibitor and ferric salt solutions were recorded individually and combined (1:1 ratio) in an equal mixture of DMSO and water using UV-Visible spectrophotometer Shimadzu UV-1800. It helped to study the metal-binding ability of Schiff base inhibitors.

***In vitro antibacterial effects of inhibitors***

*In vitro* antibacterial effects of the Schiff base inhibitors 1) *N*-hydroxy-1-(pyridin-2-yl) methanimine, NHP2M 2) *N*-hydroxy-1-(pyridin-3-yl) methanimine, NHP3M 3) (E)-2-(1-(2-phenylhydrazono)ethyl) pyridine, 2PHEP and 4) (E)-2-(1-triazylidineethyl) pyridine, 2TAEP were carried out against *Escherichia coli*. Disc diffusion method was employed for antimicrobial susceptibility tests. To compare the antimicrobial activity of inhibitors, tetracycline was used as the standard antibiotic.

❖ ***Disc diffusion method***

Disc diffusion method is also called Kirby–Bauer test[142]. This method measures the degree of resistance of pathogenic microorganisms to various antimicrobial compounds. It involves different steps.

Mueller-Hinton agar (MHA) plates were prepared using the following components[143] at 7.4 pH.

Components:

Beef extract-2 g

Acid Hydrolysate of Casein-17.50 g

Starch-1.50 g

Agar-17 g (2%)

Distilled water-1000 ml

These components dissolved in distilled water and autoclaved after closing it with

a sterilized cotton ball at $121^0$C for 15 minutes. Then cooled for half an hour and poured into a sterilized petri dish (diameter-90 mm and depth-4 mm) and placed in laminar flow hood chamber. Allowed the plate to solidify and examined each set of plates for sterility by incubating at 30-$35^0$C for 24 hrs.

For the preparation of inoculums growth method is used. From an agar plate culture, four to six isolated bacterial colonies having similar morphology was selected and then the growth is transferred to a tube consisting of nutrient broth (5 ml). Then incubation of the broth culture at $35^0$C was done until it gains the turbidity of 0.5 McFarland standards. Normally 2-6 hrs will take to produce turbidity.

Selected bacterial strain, *Escherichia coli* of 24 hrs culture, was evenly spread into the surface of agar plates using sterile swab sticks. Sterile paper discs were dipped into different samples and placed gently on the plate. Using a micropipette samples were inoculated to the discs. Discs are then incubated at $37^0$C for 24 hrs after placing it in a petri dish. The disc dipped in DMSO served as a negative control, and the tetracycline disc was acted as a positive control. Discs containing samples were allocated on the surface of the agar plate by means of forceps and then gently pressed down to attain uniform contact with agar plate. A minimum distance of 30 mm was preserved between the discs. Then the petri dishes were inverted and incubated at 37ºC for 24 hrs and observed for zone of inhibition. If the sample is resistant to the bacteria, there will be a zone around the bacteria called zone of inhibition where there is no bacterial growth. Zone of inhibition was then measured, and antibacterial activity was expressed in terms of the mean diameter of zone of inhibition in millimetres (mm) by comparing it with the standard antibiotic.

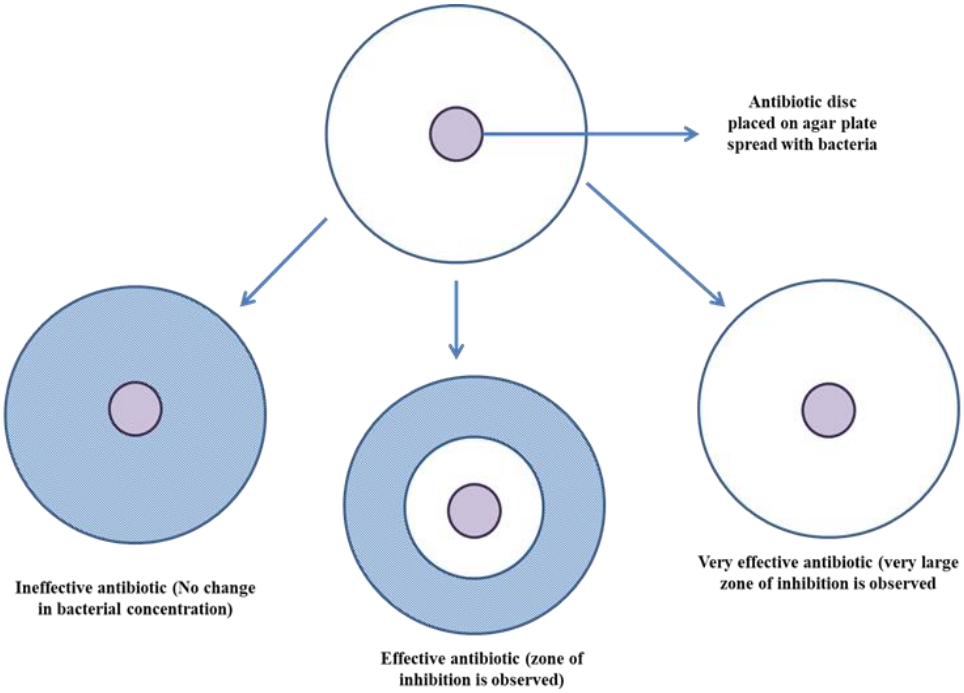



Fig. 2.6: Schematic representation of disc diffusion method