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Antimicrobial activity and corrosion inhibition property of Schiff bases derived from Imidazole

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Schiff base ligands such as methyl 4-[(2-butyl-4-chloro-5-formyl-1*H*-imidazol-1-yl)methyl]benzoate thiosemicarbazone (L_1) and 2-butyl-4-chloro-5-formylimidazole 2,4-dinitrophenylhydrzone (L_2) are designed and synthesized via the reaction between methyl 4-[(2-butyl-4-chloro-5-formyl-1*H*-imidazol-1-yl)methyl]benzoate & thiosemicarbazide for $L_1$and 2-butyl-4-chloro-5-formylimidazole & 2,4-dinitrophenylhydrazine for L_2.Schiff bases are characterized by FT-IR, UV-visible, mass spectrometry,^{1}H and ^{13}C-NMR spectral studies. These ligands are individually tested for their antimicrobial activities for both gram positive and gram negative to examine their inhibition potential by well diffusion method. The corrosion inhibition property of all the three ligands L_1, L_2 & L_3 on mild steel in 0.5 N HCl solution has been investigated at different concentrations and different temperatures by weight loss method. The biological activity of L_2 has shown better activity against gram negative bacteria such as *E-coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and gram positive bacteria *Staphylococcus aureus* when compared to standard ligand L_3. All the three ligands exhibit good corrosion inhibition property on mild steel in 0.5N HCl solution even at 0.1% concentration level and the rate of corrosion of mild steel is increased with increase of temperature of corrosion medium.

Keywords: Antibacterial activity, Butyl chloro formyl imidazole, Corrosion inhibition, Phenyl hydrazine, Schiff base ligand, Thiosemicarbazide.

The heterocyclic compounds are backbone of several biologically active compounds[1,2]. The design and study of Schiff base containing sulfur donor atom is interesting field of inorganic and bioinorganic chemistry[3-5]. Thiosemicarbazone are exhibit cytotoxic activity against a series of murine and human tumor cells in culture[6,7]. The chemistry of imidazole was reviewed in literature and found that the number of imidazole derivatives act as therapeutic agent[8] like antibacterial[9], antimalarial[10], antihypertensive[11], antidepressant[12], antitubecular[13], antiviral[14], antiepileptic[15], anti-inflammatory[16], anticancer[17] etc., Hydrazone derivatives exhibit physiological activities in the treatment of tuberculosis. They also exhibit as herbicides, insecticides, nematocides, rodenticides, plant growth regulator[18]. The nitro group is a strong electron withdrawing group and nitro Schiff bases plays an important role in affecting the reactivity and enantioselectivities in synthesis as catalyst. Recently reported Schiff bases containing O, N or S atom possess an effective corrosion inhibition for mild steel[19] and other metals[20]. The lone pair of electrons on heteroatoms in a compound has been reported to be an efficient corrosion inhibitor for metals and alloys. In an acidic environment, organic compounds with more than one heteroatom's containing electrons exhibit high corrosion inhibiting properties by providing electrons to interact with metal surface[21]. The efficient of corrosion inhibition property increases by the presence of either π-electrons of aromatic system or presence of electronegative groups (Nitro). The π-electrons facilitate the interaction with d-orbital electron of iron[22].

Losartan and Eprosartan are the antihypertensive drugs which contain buylchloroimidazole (Losartan, Allisartan) and butyimidazolechalcone (Eprosartan) skeleton illustrated in Fig. 1.

2-Butyl-4-chloro-5-formyl imidazole (BCFI) is an important intermediate used as precursors for designing a variety of antihypertensive drugs. It was reported that butylchloroimidazole derivatives are gained synthetic interest in recent years since it exhibits broad spectrum of biological properties[23]. Also butyl chloro imidazole chalcones and pyrazoles act as good inhibitors for angiotensin converting enzyme[24].The nitro benzyl 2-butyl-4-chloroimidazole derivative act as anti-inflammatory and analgesic activity[25]. The nickel complex of Schiff base derived

from BCFI and ethylenediamine showed excellent activity against *E. coli* bacteria[26].

By keeping all the above facts in mind, in the present work we made an effort to design and synthesis a new butyl chloro imidazole Schiff base ligands illustrated in Fig. 2. The synthesis of standard ligand L_3 has been described[27]. The L_1 has been designed by protecting the imidazole –NH group of

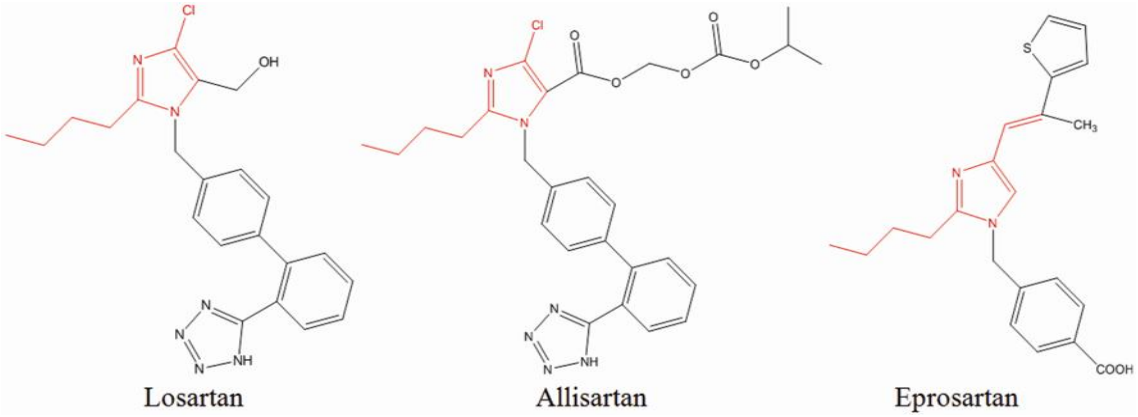


Fig. 1 — Structure of drugs containing butylchloroimidazole skeleton.

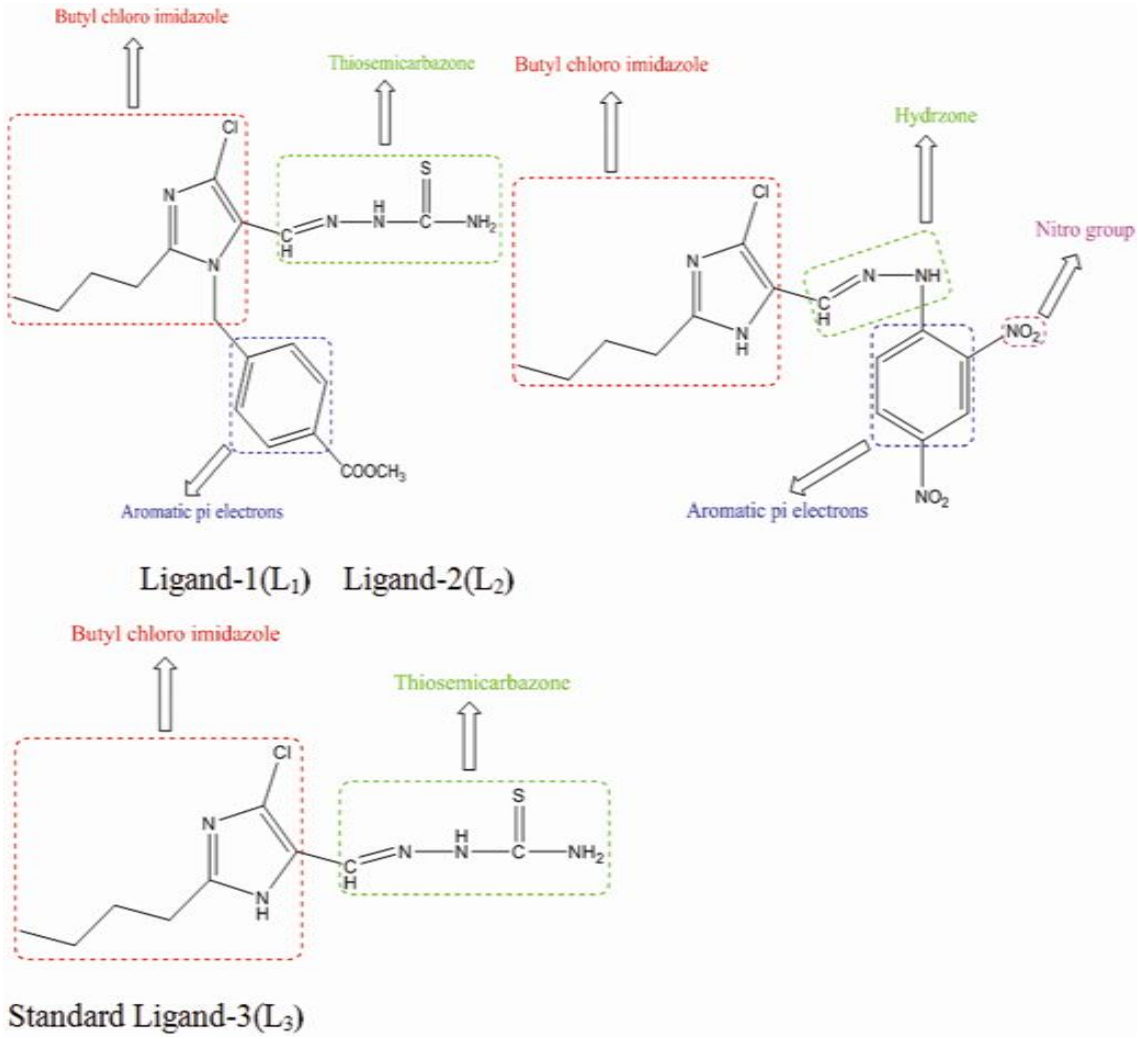


Fig. 2 — New Schiff base design strategy

standard L_3 with benzyl-4-methylbenzoate ester group and the L_2 has been designed by replacing the amine part of standard L_3 with 2,4-dinitrophenylhydrazine. The antimicrobial studies against gram positive and gram negative pathogens and corrosion inhibition property for mild steel in 0.5N HCl at different concentrations and temperatures were investigated.

Experimental Section

Materials and methods

All chemicals and solvents used in the present work were of analytical grade. 2-butyl-4-chloro-5-formyl imidazole and methyl 4-[(2-butyl-4-chloro-5-formyl-1H-imidazol-1-yl)methyl]benzoate were purchased from sigma-Aldrich. Thiosemicarbazide, 2,4-dinitrophenylhydrazine, were purchased from SD fine chemicals. Acetic acid, concentrated hydrochloric acid, methanol, ethanol was purchased form Merck.

Instrumentation

The electronic spectra of the compound were recorded using a PerkinElmer LS 45 UV-Visible spectrophotometer. The IR spectrums were recorded by using JASCO FT/IR-6300 attenuated total reflection Fourier transform infrared spectrometer (ATR-FTIR). The mass spectrums were recorded by using of mass spectrometer Jeol GC MATE-II EI mass equipped with energy of 70ev. The NMR spectrum is recorded on a Bruker (400MHz) spectrometer at 297.7 K, using DMSO-d_6 and $CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal reference compound.

Synthesis of Ligand-1(L_1)

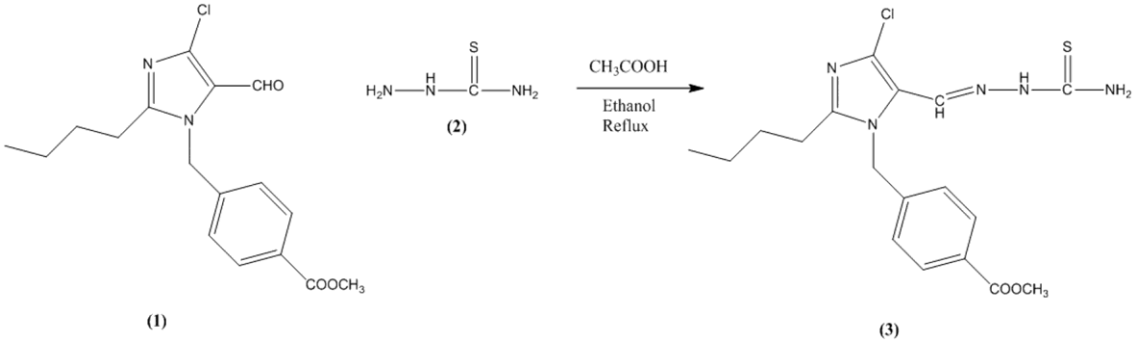
Methyl 4-[(2-butyl-4-chloro-5-formyl-1H-imidazol-1-yl)methyl]benzoate(1)(10mmol;1g) in ethanol(10 ml) was added to thiosemicarbazide(2)(10mmol;0.27g) in ethanol(10ml). 5-10 drops of acetic acid was added. The reaction mixture was refluxed for about 1h. The progress of the reaction was monitored by TLC (1:1 ethylacetate+hexane system) and cooled to room temperature. The white precipitate formed was filtered and washed with ethanol. The solid was dried under vacuum at room temperature to yield the L_1 (3) and recrystallized from hot ethanol. The reaction scheme is shown in Scheme-1.

Yield : 85%, Description: white powder, HRMS(m/z): Calcd for $[C_{18}H_{22}O_2N_5S_1Cl_1]^+$ is 407.9 Found: 407.89, IR(cm^{-1}): γ(N-H) 3398, γ(NH_2) 3248, γ(C=N) 1605, γ(C=S) 795, γ(N-N) 1102, γ(C=O, ester) 1727, γ(C-Cl) 843, γ(aliphatic, aromatic C-H) 2947, γ(aromatic C=C) 1532, 1462. ^{1}H NMR(d_6-DMSO)(ppm) : ∂=0.81 (3H, t, butylCH_3), ∂=1.22-1.28(2H, m, butylCH_2), ∂=1.46-1.52(2H, m, butylCH_2), ∂2.6-2.63(2H, t, butylCH_2), ∂=3.82(3H, s, esterCH_3), ∂=5.7 (2H, s, N-CH_2), ∂=8.02(1H, s,-NH_2), ∂8.09(1H, s, -NH_2), ∂=6.9(1H, s, -CH), ∂=7.10-7.12(2H, d, AroCH), ∂=7.89-7.91(2H, d, AroCH), ∂=11.27(1H, s, -NH).^{13}C NMR (d_6-DMSO) (ppm): ∂=14.0, 22.0, 26.0, 29.4, 48.4, 52.5, 120.8, 126.4, 129.0, 130.0, 132.3, 133.3, 143.1, 152.1, 166.3, 177.6.

Synthesis of Ligand-2(L_2)

2-Butyl-4-chloro-5-formylimidazole (4) (10m moL;1g) in methanol (10mL) was added to 2,4-dinitrophenylhydrazine (5) (10m mol;1.06 g) in methanol (10 mL) followed by the addition of 5-10 drops of concentrated hydrochloric acid. The reaction mixture was stirred at room temperature about 3-4 H. The progress of the reaction was monitored by TLC (20%Ethyl acetate in hexane system). The red precipitate obtained was filtered and washed with methanol and then dried the solid under vacuum at room temperature to yield the L_2 (6). The solid was recrystallized from hot ethanol.The reaction scheme is shown in Scheme 2.

Yield : 87%, Description: Red orange powder, HRMS(m/z): Calcd for$[C_{14}H_{15}O_4N_6Cl_1]^+$ is 366.7 Found: 366.69, IR(cm^{-1}): γ(N-H) 3276, γ(NO_2) 1511,



Scheme 1 — Preparation of L_1

γ(C=N) 1612, γ(N-N) 1138, γ(C-Cl) 839, γ(aliphatic, aromatic C-H) 3106 & 2960, γ(aromatic C=C) 1423. ^{1}H NMR($CDCl_3$) (ppm): ∂=0.95-0.98(3H, t, butylCH_3), ∂=1.38-1.48(2H, m, butylCH_2), ∂=1.74-1.82(2H, m, butylCH_2), ∂=2.76-2.79(2H, t, butylCH_2), ∂=7.2-8.3(2H, AroCH), ∂=9.15(1H, s, -CH), ∂=9.46(1H, s, -NH), ∂=11.3(1H, s, Imidazole-NH). ^{13}C NMR(DMSO) (ppm):14.0, 22.1, 28.1, 30.2, 117.4, 123.5, 129.5, 129.8, 137.2, 137.6, 144.8, 151.7.

Synthesis of Ligand-3(L_3)

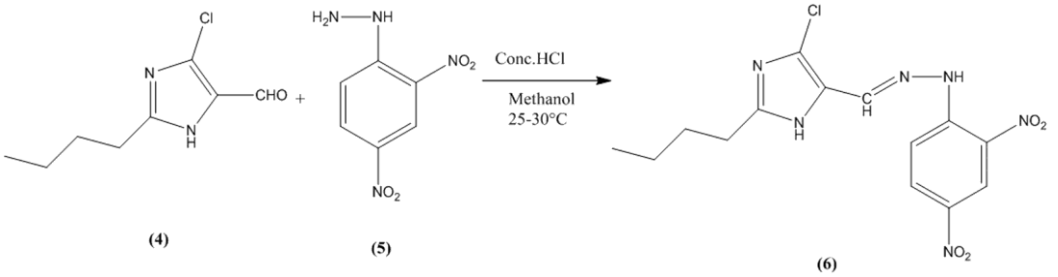
2-Butyl-4-chloro-5-formylimidazole (7) (10mmol; 1g) in ethanol (10 mL) was added to thiosemicarbazide (8) (10mmol;0.49g) in ethanol (10 mL) followed by 5-10 drops of acetic acid was added. The reaction mixture was refluxed for about 1h. The progress of the reaction was monitored by TLC (1:1 Ethylacetate+hexane system). The reaction mixture was cooled to room temperature and the white precipitate formed was filtered and washed with ethanol. The solid was dried under vacuum at room temperature to yield the L_3 (9). Then it was recrystallized from hot ethanol. The reaction scheme is shown in Scheme 3.

Yield : 84%, Description: white powder, HRMS (m/z): Calcd for$[C_9H_{14}N_5S_1Cl_1]^+$ is 259.7 Found: 259.1, IR (cm^{-1}): γ(N-H) 3353, γ(NH_2) 3258, γ(C=N) 1606, γ(aliphatic, C-H) 3171 & 2925, γ(N-N) 1244, γ(C-Cl, C=S) 817, γ(aromatic C=C) 1477 & 1509.^{1}H NMR (ppm) : ∂=0.88-0.91(3H, t, butylCH_3), ∂=1.30-1.32 (2H, m, butylCH_2), ∂=1.61-65(2H, m, butylCH_2), ∂=2.59-2.63(2H, t, butylCH_2), ∂=7.83(1H, s, -NH_2), ∂7.95(1H, s, -NH_2), ∂=8.33(1H, s, -CH), ∂=11.45(1H, s, N-NH).^{13}C NMR(d_6-DMSO) (ppm): ∂=14.0, 22.1, 28.2, 30.1, 79.6, 122.1, 129.1, 129.9, 150.4, 178.2.

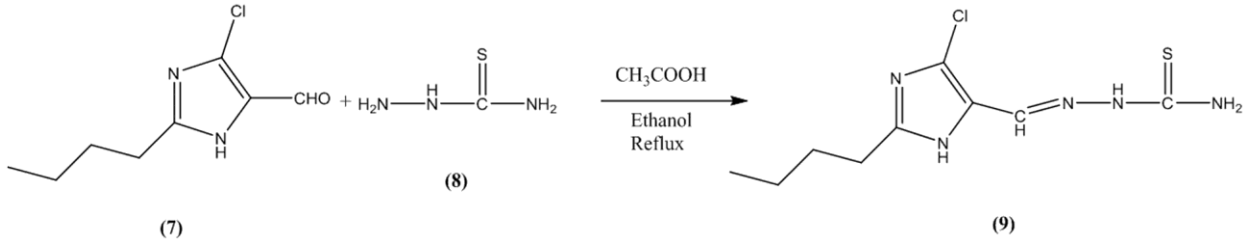
Antimicrobial activity

The agar well diffusion method was used for examine the *in vitro* antimicrobial activity of synthesized ligands L_1, L_2 & L_3. The antimicrobial activity of synthesized ligands were screened against both gram positive organism such as *Staphylococcus aureus* and gram negative organisms such as *E-coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* with the quantity of 100µl of concentration of 1% solution (10 mg of testing ligand was dissolved in 1mL of dimethylsulfoxide).

The microorganisms were inoculated into the sterilized nutrient broth and it was maintained at 37°C about 24 h. On the day of testing, 100 µL of exponentially grown inocula of *E.coli*-ATCC 25922, *Staphylococcus aureus* - MTCC 1430, *Klebsiella pneumoniae* -MTCC 432, *Acinetobacter baumannii*-ATCC 19606, *Pseudomonas aeruginosa* -ATCC 27853 were uniformly spread with the help of L-rod on nutrient agar plates. After even spreading of all cultures, 6 mm diameter wells were cut on nutrient agar plates and 100 µL of ligands L_1, L_2 & L_3 were transferred into all the wells immediately. 100 µL DMSO is spotted separately to compare the effect of solvent on antimicrobial activity. The loaded plates were incubated at 37°C about 24h. The measurement of zone of inhibition was determined as radius of the clear zone with the help of antibiotic measuring zone scale (Himedia).



Scheme 2 — Preparation of L_2



Scheme 3 — Preparation of L_3

Corrosion study

The experiment was performed on mild steel with the chemical composition of iron-99.099% and carbon-0.084%. The size of rectangular mild steel specimens are 5cm × 2cm × 0.05cm (length × width × thickness) with a small hole at one end. The specimens were polished with 4/0,3/0,2/0 and 1/0 grade emery papers to remove dusts or other foreign matter followed by rinsing thoroughly with double distilled water degreased with acetone and dried.

The initial weight of the specimens was measured. A series of 200 mL 0.5N hydrochloric acid solutions were prepared using double distilled water. The prior weighed specimens are completely immersed into the solution through glass hook. The temperature was maintained 30±2°C about 24 h. The specimens were removed from the solution, washed with running water, dried and weighed to the accuracy of four decimal places. The weight loss of mild steel was calculated by the difference between the weight of mild steel before and after immersion. The experiment was repeated for the different concentrations of L_1, L_2 & L_3 with 0.1, 0.3, 0.5 and 0.7% respectively. To derive the effect of temperature on corrosion inhibition of mild steel, the above procedure was carried out at 40±2 and 50±2°C.

Result and Discussion

Mass spectra

The mass (m/z) is obtained for L_1, L_2& L_3 are 407.89, 366.69 & 259.1 respectively which are well supporting the proposed molecular mass calculated from molecular formula for these ligands. Along with molecular ion peak, the spectra exhibit some other peaks which are assignable to various possible fragments[28].

Infra-Red spectra

The synthesized new ligands L_1 & L_2 and standard ligand L_3 shows a significant vibrational bands corresponding to the characteristic functional groups in the molecules. The strong evidence for the formation of imine(C=N) was found (the sharp and strong stretching vibrational band) at 1605 cm^{-1} (for L_1), 1612 cm^{-1} (for L_2) and 1606 cm^{-1} (for L_3) and the absence of carbonyl (C=O) group stretching vibrational band at 1700cm^{-1} for L_1, L_2 & L_3. Further, the stretching vibrational band at 1727cm^{-1} and 3248 cm^{-1} confirms the presence of ester(-$COOCH_3$) and amine(-NH_2) group in L_1.The stretching vibrational band at 1511 cm^{-1} and 839 cm^{-1} confirms the presence of nitro(-NO_2) and C-Cl bond in L_2[29]. The stretching vibrational band at 3258cm^{-1} and 817 cm^{-1} confirms the presence of amine(-NH_2) and C-Cl bond in L_3.

Ultraviolet-Visible spectra

The UV spectra of L_1,L_2 & $L_3$is showed an intense broad peak at 335nm (for L_1& L_3) and 418nm (for L_2) due to π -π^*transition in azomethine group(-C=N)[30]. The conjugation of double bond in the imidazole ring shifted the wavelength to lower region by the bathochromic shift. The probable forbidden n-π^* is partially overlapped by the allowed π -π^* for L_1& L_3. The short band at 305nm is due to n-π^*transition azomethine group for L_2[31].

NMR spectra

The ^{1}H NMR spectrum of the synthesized ligands confirms the structure of the molecule. For L_1, the thioamide protons have shown the chemical shift values of ∂=8.03 and 8.09 as two different singlet due to the involvement of one proton in hydrogen bonding with imine nitrogen. The secondary amine proton is observed as singlet (broad peak) at ∂ value of 11.2. The imine –CH and methylene (-CH_2) attached to imidazole ring is shown as singlet at ∂ value of 6.9 and 5.7 respectively[32]. The aliphatic protons on the n-butyl side chain attached to the imidazole ring are found at ∂ value of 0.81, 1.2, 1.4 and 2.6. The aromatic protons are observed as doublet at ∂ value between 7.1-7.9. For L_2, the imine –CH is found at ∂ of 9.15 as singlet. This is due to the deshielding effect of presence of dinitrophenyl group is shift to higher ∂ value. The imidazole –NH proton is appeared as singlet at ∂ value of 11.3[33]. The aliphatic protons on butyl side chain are observed at ∂ value of 0.95, 1.3, 1.7 and 2.7 respectively. The aromatic protons on phenyl ring are observed at ∂ value of 7.2-8.3. For L_3, the thioamide protons have shown the chemical shift values of ∂=7.95 and 8.33 as two different singlet. The imine –CH is shown as singlet at ∂ value of 7.83. The imidazole –NH proton is appeared as singlet at ∂ value of 11.4. The aliphatic protons on butyl side chain are observed at ∂ value of 0.98, 1.3, 1.6 and 2.6 respectively. The ^{13}C NMR spectrum of L_1, L_2 & L_3 are further confirmed the formation of imine group in the molecule. The imine carbon was shown the peak at ∂ value of 130.0, 129.8 and 129.9 for L_1, L_2 & L_3 respectively[34]. The n-butyl side chain carbon atoms showed the peak at ∂ value of 14.0, 22.0, 26.0, 29.4 for L_1, 14.0, 22.1, 28.1, 30.2 for L_2 and 14.0, 22.1, 28.2, 30.2 for L_3.

Antimicrobial activity

The presence of nitro group (-NO_2), secondary amine group (-NH) or phenolic hydroxyl group (-OH)

in the compound which enhances the antimicrobial activity[35]. It can be seen from the data that the biological study of synthesized new ligand L_1 (imidazole –NH of L_3 is protected with aromatic substituent) has shown almost equal activity when compared to standard ligand L_3 whereas the another new ligand L_2(amine part of L_3 is replaced with 2,4-dinitrophenylhydrazine) has shown slightly better biological activity against gram negative bacteria such as *E-coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa and* gram positive bacteria *Staphylococcus aureus* when compared to standard ligand L_3.The ligand bearing aromatic ring and hetero atom nitrogen has shown promising activity against all the tested fungi[58]. Hence, the presence of aromatic ring with hetero atom as key atom (nitro phenyl group) enhanced the antimicrobial activity than the standard ligand L_3[36].The values of zone of inhibition of ligands L_1,L_2 & L_3 is tabulated in Table 1.

Corrosion Study

The schiff base containing the nitro group showed an effective corrosion inhibiton property on mild steel in 0.5M HCl medium by weight loss method[37]. The present corrosion study at different concentration of ligands reveals that the corrosion inhibition property was increased or weight loss and corrosion rate are decreased drastically by doping with 0.1% of ligands with 0.5N hydrochloric acid solution. Further, by increasing the concentration of ligands from 0.1% to 0.7%, the corrosion inhibition efficiency is increased from 87.57 to 89.74% for L_1, 89.71 to 91.76% for L_2 and 86.22 to 88.64% for L_3.This might be explained by the donor-acceptor interaction between the unshared pair of electrons of donor atom of the schiff bases and metals in the steel. The higher corrosion inhibition property of schiff base ligands L_1, L_2 was due to the increase of electron density in L_1 by the presence of aromatic π-electrons and ester group, where as in L_2 by the presence of nitro group and aromatic π-electrons[38]. The observed values are tabulated in Table 2. The presence of heteroatoms such as N,O or S in the compound which enhances the corrosion inhibition property in an acidic environment and the corrosion inhibition efficiency is decreased with increase of temperature[39]. The corrosion study at different temperature with concentration of 0.1% of synthesized ligands reveals that the weight loss and corrosion rate are increased by increasing the temperature from 30±2 to 50±2°C and the corrosion inhibition efficiency is decreased from 87.6 to 86.9% for L_1, 89.7 to 89.3% for L_2 and 86.5 to 86.0% for L_3 by increasing the temperature. The observed values are tabulated in Table 3.

From the weight loss measurements, the percentage of Inhibition efficiency (η), Corrosion rate(R) is calculated by the following formulae.

Table 1 — Zone of inhibition values for ligands L_1, L_2 & L_3

Human pathogens	Quantity (µL)	Zones of inhibition (mm)			
		Blank	Lig-1	Lig-2	Lig-3
E.coli-ATCC 25922	100	1	15	20	14
Staphylococcus aureus - MTCC 1430	100	5	13	18	15
Klebsiellapneumoniae -MTCC 432	100	3	18	22	18
Acinetobacterbaumannii- ATCC 19606	100	4	16	21	17
Pseudomonas aeruginosa-ATCC 27853	100	5	14	19	15

Table 2 — Corrosion Inhibition Efficiency and Corrosion rate of ligands in 0.5N HCl at 30±2°C

Inhibitor	Concentration (%)	Weight loss (mg)	Inhibition efficiency η (%)	Corrosion rate R ($mgcm^{-2}h^{-1}$)
Blank	-	82.9345	-	0.3456
Ligand-1	0.1	10.3123	87.57	0.0430
	0.3	9.2535	88.84	0.0386
	0.5	8.7321	89.47	0.0364
	0.7	8.5122	89.74	0.0355
Ligand-2	0.1	8.5321	89.71	0.0356
	0.3	7.6132	90.82	0.0317
	0.5	7.0211	91.53	0.0293
	0.7	6.8325	91.76	0.0285
Ligand-3	0.1	11.4321	86.22	0.0476
	0.3	10.3652	87.50	0.0432
	0.5	9.8652	88.10	0.0411
	0.7	9.4215	88.64	0.0393

Table 3 — Effect of temperature on corrosion inhibitor of concentration – 0.1%

Inhibitor	Temperature (°C)	Weight loss (mg)	Inhibition efficiency η (%)	Corrosion rate R ($mgcm^{-2}h^{-1}$)
Blank	30±2	82.9345	-	0.3456
	40±2	163.2152	-	0.6801
	50±2	201.3524	-	0.8390
Ligand-1	30±2	10.3123	87.57	0.0430
	40±2	21.1532	87.04	0.0881
	50±2	26.3581	86.91	0.1098
Ligand-2	30±2	8.5321	89.71	0.0356
	40±2	17.0213	89.57	0.0709
	50±2	21.5310	89.31	0.0897
Ligand-3	30±2	11.2325	86.46	0.0468
	40±2	22.5212	86.20	0.0938
	50±2	28.1321	86.03	0.1172

1. Inhibition Efficiency(η) = $[(W_{Corr}-W_{Inhi})/W_{Corr}]*100$
   Where, W_{Corr}= Weight loss without inhibitor.
   W_{Inhi} = Weight loss with inhibitor.
2. Corrosion Rate (R) = Weight loss in mg /[Area of specimen(cm^2) * Time (Hr)]

Conclusion

Schiff base L_1 & L_2, standard ligand L_3 are prepared and the structure was confirmed by UV-Visible, NMR, Mass and IR spectral studies. The new L_1 and L_2 are designed by protecting the imidazole –NH group of $L_3$with benzyl-4-methylbenzoate (for L_1) and varying the amine part of L_3 with 2,4-dinitrophenyl hydrazine (for L_2).
The antimicrobial study by well diffusion method is performed for L_1, L_2 & L_3 and the results are compared with standard ligand L_3.

Corrosion inhibition property on mild steel in 0.5N HCl for L_1 & L_2 and standard ligand L_3 has been studied by weight loss method at different concentration and different temperature.

Similar biological activity is observed by protecting the imidazole –NH with ester substituted phenyl derivative (L_1) and little improvement in biological activity is observed by the amine part is replaced with nitro substituted phenyl hydrazine derivatives(L_2) when compared to standard ligand(L_3). All the ligands exhibit excellent antimicrobial activity against gram negative bacteria *E-coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and gram positive bacteria *Staphylococcus aureus.*

All the ligands exhibit as good corrosion inhibitor on mild steel in 0.5N HCl medium. The corrosion inhibition efficiency (η) is increased gradually and corrosion rate (R) is decreased gradually by increasing the concentration of ligands from 0.1 to 0.7%. The corrosion study at elevated temperature reveals that, the corrosion inhibition efficiency (η) is decreased whereas corrosion rate (R) is increased by increasing the temperature from 30±2 to 50±2°C.

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