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Kinetics of Rapid Chlorination of Imidazole by N-Chlorosuccinimide in Aqueous Medium and Study of Antimicrobial Activity of its Chloro Derivative

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A B S T R A C T

The rapid kinetics of the chlorination of imidazole by N-chlorosuccinimide (NCS) has been studied using hydrodynamic voltammetry technique in aqueous medium. The specific reaction rate at the temperature 301.6 K is found to be $5.48M^{-1}s^{-1}$. The mono chloro derivative formed in the reaction is confirmed stoichiometrically and by ^{1}H NMR spectral study. It was screened for antifungal and antibacterial activity against the different bacteria and fungi species by agar well diffusion method. The results of antibacterial and antifungal activity revel that the chloro derivative of imidazole acts as a potential antifungal and antibacterial agent.

Keywords: Kinetics, imidazole, hydrodynamic voltammetry, antimicrobial activity, agar well diffusion method.

A R T I C L E I N F O

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1. Introduction

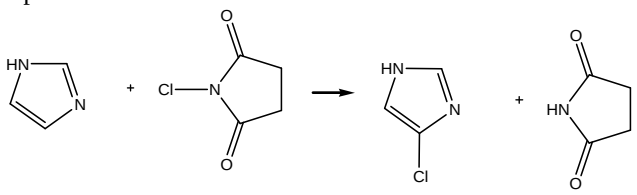
Imidazole is a heterocyclic compound which contains 5-membered planar ring. Presence of Π electrons makes the imidazole as aromatic compound. It is incorporated in many important biological molecules. The substitution in imidazole makes number of compounds of interest. These substituted derivatives are important in organic synthesis as they serve

building blocks for the construction of biologically important molecules [1]. The derivatives of imidazoles in recent years have attracted interest for their versatile properties in Chemistry and Pharmacology. It has been noticed that the modification on imidazole nucleus displayed promising biological activities [2]. These derivatives have been used in the drug discovery [3, 4]. Many substituted derivatives are selective inhibitors of nitric oxide synthase, which makes them interesting drug targets in inflammation, neurodegenerative diseases and tumors of the nervous system [5, 6]. Imidazole belongs to the class of azole antifungal, which includes ketoconazole, miconazole and clotrimazole. In treatment of many systemic fungal infections, the imidazole derivatives have been used [7]. Many workers have reported the antimicrobial and antifungal activity of imidazole and its various derivatives [8-13].

The anti inflammatory activity has also been reported by earlier investigators [14]. The recent advancement in imidazole revel that imidazole derivatives shows activity against the cancer [15]. The pharmacological activities such as anticonvulsant [16], anti-Parkinson [17] and mono-aminooxidase (MAO) inhibitory activity [18] have also been reported previously. Imidazole rings widely employed as spin –trapping species in the interesting application of designing drugs with neuroprotective activity [19]. It has been observed that the imidazole based derivatives have considerable development potential in medicinal chemistry due to the several favorable properties such as excellent bioavailability, good tissue penetrability and permeability and a relatively low incidence of adverse and toxic effects [20]. These enormous applications associated with imidazole derivatives prompted researchers to work in this area.

Presently we have studied the kinetics of chlorination of imidazole by N-chlorosuccinimide (NCS) in aqueous medium. The product of which is 4-chloroimidazole confirmed stoichiometrically and by ^{1}H NMR spectral analysis. It has been observed that chlorination of imidazole is rapid one, having half life about 60 seconds. The product thus obtained instantly, which has been screened for the antimicrobial activity. The rapidity of this reaction is the advantage in the present study. The antibacterial activity is tested against some gram-negative bacteria such as *Escherichia coli* (NCIM 5010) *and Psedumonas aeruginosa* (NCIM 2862) and some gram-positive bacteria such as *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2079).

The antifungal activity of 4-chloroimidazole is also tested against various fungi such as *Candida albians* (NCIM 3100), *Saccharomyces cerevisiae* (NCIM 3176), *Aspergillus niger* (NCIM 545), *Penicillium notatum* (NCIM 741), *Aspergillus fumigates* (NCIM 902), *Penicillium chrysogenum* (NCIM 709) and *Penicillium chrysogenum* (NCIM 723). The agar well diffusion method is employed for this study. The reaction is observed to be too rapid to study by conventional methods. The special technique, hydrodynamic voltammetry has been employed in which rotating platinum electrode (RPE) is used to monitor the decaying concentration of NCS [21]. Kinetics of several rapid halogenations has been studied by using RPE [22-24]. The ^{1}H NMR spectral analysis shows that the substitution takes place at position 4 and hence the 4-chloroimidazole formation is confirmed. The reaction under study is represented as:



2. Materials and Methods

Kinetic study material

Imidazoles, N-chlorosuccinimide, potassium nitrate of analytical grade chemicals were used in present study to prepare the stock solutions of required concentrations. The solutions of required concentration were prepared in double distilled water. Solution of N-chlorosuccinimide was prepared with hundred folds potassium nitrate as supporting electrolyte.

Calibration of diffusion current

Calibration of the diffusion current was carried out with the help lamp and scale arrangement. The galvanometer light spot was adjusted to zero deflection on the scale with the help of shunt using 100 fold potassium nitrate solutions. The maximum deflection of light spot at 35 cm was adjusted for the 2.5× 10^{-3}M N-chlorosuccinimide. The diffusion current was measured in terms of galvanometer deflection on scale in centimeter unit, which was converted then in nano-ampere (nA) unit. The diffusion current values were recorded at various known concentrations of NCS in the range of 0.5 × 10^{-3} to 2.5 ×10^{-3} M, the data is given in Table 1. The plot of diffusion current Vs concentration of NCS was found to be linear as shown in Figure 1.

Table 1: Calibration of the diffusion current

[NCS] (10^{-3} M)	Diffusion current (nA)
0.5	8.0
1.0	13.7
1.5	20.8
2.0	26.2
2.5	35.0

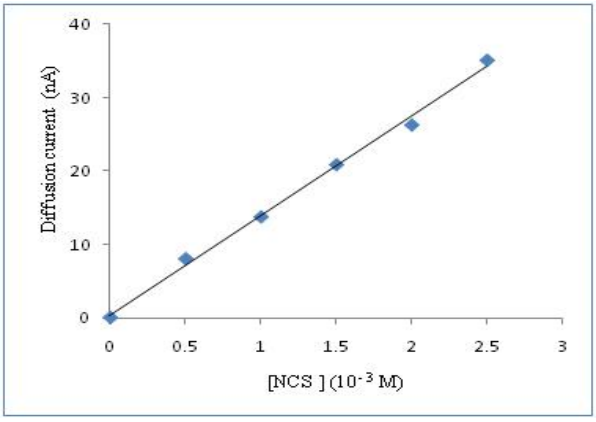


Figure 1: Calibration of diffusion current

Kinetic measurements

Equimolar solutions of imidazole and NCS containing 100 folds potassium nitrate were poured simultaneously in a beaker assembled with RPE and SCE after attaining the thermostat temperature. At the moment of mixing a stopwatch was started. The extent of reaction was measured by recording the diffusion current at various time intervals. The concentration of unreacted NCS at this time intervals was determined from the calibration curve (Table 2). The above procedure of calibration and kinetic measurement was repeated twice for checking the reproducibility of the diffusion current, and these were found to be within the limits ±0.2 nA. The reciprocal of [NCS] versus time was plotted (Fig. 2). A straight line was obtained since the reaction is of second order; the slope of this plot gives specific reaction rate k_2.

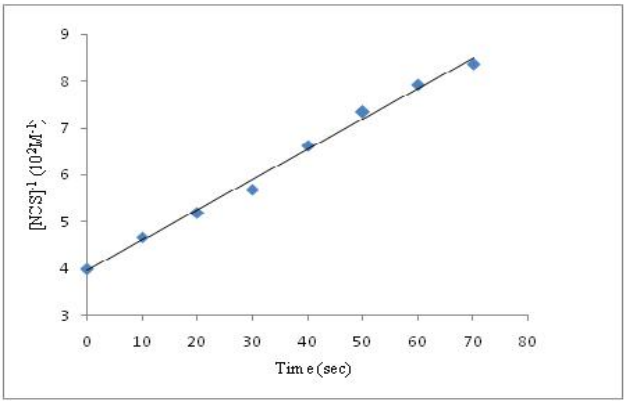


Figure 2: Kinetics of chlorination of imidazole using NCS in aqueous medium at 301.6 K

Antimicrobial activity study

Preparation of microbial cultures

The microbial strains of Gram-positive bacteria *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2079), Gram-negative bacteria *Escherichia coli* (NCIM 5010), *Psedumonas aeruginosa* (NCIM 2862) were used for assay. Cultures were obtained from NCL, Pune, were separately enriched by culturing for 24 hrs at 37^{o}C in universal culture medium Nutrient broth (Hi-media laboratory Pvt Ltd Mumbai). Then using the broth culture dilutions of each culture containing 1×10^{8} CFU/ml were prepared and used in the antibacterial test.

Antibacterial activity assay

Prepared cultures of *Escherichia coli* (NCIM 5010), *Psedumonas aeruginosa* (NCIM 2862), *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2079) were used for this assay. Antibacterial activity of the product viz. 4-chloroimidazole obtained from the above kinetic study was carried out by agar well diffusion method [25]. The media used here nutrient agar (Hi-Media Pvt Ltd Mumbai) for checking antibacterial activity. The media was prepared by dissolving the agar Medium in distilled water. The dissolved media was autoclaved at 15 lbs pressure and 121 ^{o}C. It was then mixed well and poured into the petri-dishes while still molten. From each enriched bacterial cultures 100 µL were spreaded on nutrient agar plate (1×10^{8} CFU/ml). Then with the help of sterile cork borer well of 6 mm in diameter were prepared at the center of each plate. 100 µl of solution of 4-chloroimdazole was separately added to wells in plates which were inoculated with each bacteria. Then the plates were incubated at 37 ^{o}C for 24 hrs. After incubation, diameters of zone of inhibition were measured in mm. Antibacterial activity of 4-chloroimidazole against two Gram positive and two Gram negative bacteria were evaluated using these diameters of zone of inhibition. The antimicrobial study has been carried out at various parameters such as different pH (pH= 3.5, 5.0, 7.4, 8.0 and 10), different temperatures (t / ^{o}C = 8, 22, 37 and 45), different concentrations (C/µg/ml = 16, 32, 64, 128 and 256). Reference antibacterial compound ciprofloxacin (10 µg/mL) was used as positive control for comparison. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of diameter of zones of inhibition in mm.

Preparation of fungal cultures

The antifungal activity of the product obtained from the above kinetic study namely 4-chloroimdazole was evaluated against the various fungi namely *Aspergillus niger* (NCIM 545), *Penicillium notatum* (NCIM 741), *Aspergillus fumigates* (NCIM 902), *Penicillium chrysogenum* (NCIM 709), *Penicillium chrysogenum* (NCIM 723) and yeasts such as *Candida albians* (NCIM 3100), *Saccharomyces cerevisiae* (NCIM 3176). The fungal inoculums consisted of aqueous spore suspension obtained from 4 days old culture Czapek Dox agar plates incubated spores were harvested by flooding plates with 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80, and passing the suspension through double-layered sterile cheesecloth to remove hyphal fragments [26]. The spore concentration was determined with the aid of haemocytometer and adjusted to 10^{6} spores per ml with sterile water.

Antifungal activity assay

The antifungal activity was carried out by agar well diffusion method [27]. Inoculums (100 µl) of respective fungal and yeast species were used for inoculation of fungal and yeasts strains on PDA plates. An aliquot of inoculums was introduced to molten PDA and poured in to a petri-dish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using sterile cork borer. In agar well diffusion method 100µl extracts of solution of 4-chloroimidazole was introduced serially. Incubation period of 48-72 hours at 28 ^{o}C was maintained for observation of antifungal activity of 4-chloroimidazole. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the extracts. The complete antifungal analysis was carried out under strict aseptic conditions. Reference antifungal compound Ketoconazole (10 µg/ml) was used as positive control for comparison. The experiment was carried out in triplicates and the antifungal activity was expressed as the mean of diameter of zones of inhibition in mm.

3. Results and Discussions

Kinetic measurements

The decrease in diffusion current was obtained on addition of the imidazole solution to the solution of N-chloro-succinimide. The linear plot is obtained for $[NCS]^{-1}$ against

time confirmed the second order kinetics. Thus, the rate of this reaction is given as:

$$\text{Rate} = k_2 [\text{Imidazole}] [\text{NCS}]$$

Where k_2 is the second order rate constant, which is obtained from the slope of the above plot. The value of the rate constant obtained as 5.48 $M^{-1}s^{-1}$ at 301.6 K The observed rate constant for the reaction studied shows the rapidity of the reaction which necessitates the use of special technique namely, hydrodynamic voltammetry. The product obtained for the above kinetic study is confirmed stochiometrically and by predicting the ^{1}H NMR spectrum. (Fig. 3) The appearance of singlet around 7.65 ppm (1H) at C-2 and another singlet at 7.25 ppm (1H) at C-3 indicates the formation of mono chloro derivative. Also the ^{1}H NMR spectra confirms the substitution takes place at position 4 and the product obtained is thus the 4-chloroimidazole.

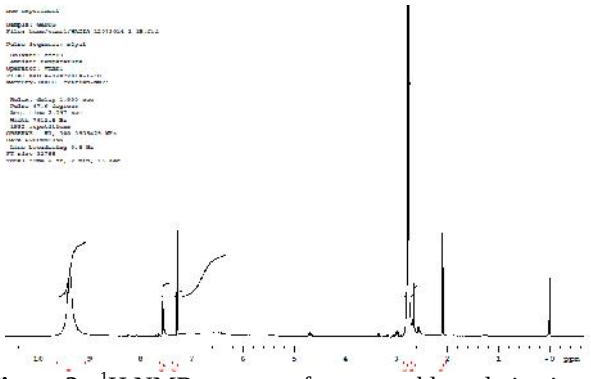
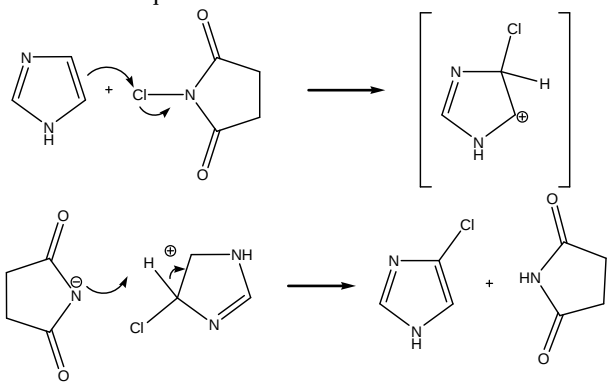


Figure 3: ^{1}H NMR spectrum for mono chloro derivative of imidazole

The reaction takes place via electrophilic substitution at C-4. The polarization of N-Chlorosuccinimide takes place in polar solvent to generate electrophile. In slow step the +ve end of reagent $Cl^{\delta+}$ is then attacks the Π electrons of the aromatic imidazole ring which results in formation of carocation and loss of aromaticity. In fast step, the carbocation intermediate is attacked by the base and loses the proton. These electrons are used to reform a pi bond and restore aromaticity. The proposed mechanism may be represented by scheme 1. It is evidenced that the electophilic attack of NCS on the imidazole takes place at C-4 is only kinetically significance process in the aqueous chlorination of imidazole



Scheme 1: Plausible mechanism of chlorination of imidazole by N-chlorosuccinimide

The product obtained in this reaction is having very low concentration since the concentration of reactants is low. From the kinetic study though the product is not obtained on a large scale, but it is observed that it acts as an antibacterial and antifungal agent. In the drug discovery the imidazole derivatives are the most important synthetic strategy. A lot of work has been done and a lot to do in this field.

Antimicrobial assay:

The search for the antifungal drugs in recent years has concentrated principally on the imidazole and its derivatives. The group of drugs comprising imidazole represents the modern approach to both topical and systematic treatment of fungal diseases. This low concentrated product solution of 4-chloroimidazole when tested for its antimicrobial activity the study reveals that it has a potential antibacterial and antifungal activity.

Antibacterial activity assay

The results of antibacterial study at various parameters were tabulated in Table 3 to Table 6. The results show that the product exhibits antibacterial activity against the Gram negative bacteria such as *Escherichia coli* (NCIM 5010) and for *Pseudomonas aeruginosa* (NCIM 2862) while it fails to exhibit the antibacterial activity against the Gram positive bacteria such as *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2079). The product is highly active against the *Pseudomonas aeruginosa* (NCIM 2862) species compared to *Escherichia coli* (NCIM 5010). It is thus screened for its antibacterial activity against the *Pseudomonas aeruginosa* (NCIM 2862) at various parameters such as pH, temperature and concentrations to check its optimum activity conditions. The results show that the product exhibits the maximum antibacterial activity at universal pH and temperature against *Pseudomonas aeruginosa* (NCIM 2862). The concentration variation study shows that the product at concentration 16 µg/ml exhibits less antibacterial activity while for concentration 256 µg/ml more antibacterial activity against *Pseudomonas aeruginosa* (NCIM 2862). The overall result reveals that the product exhibits maximum antibacterial activity at 37.0 ^{o}C, at pH 7.4 and at concentration 256 µg/mL

Table 3: Antibacterial activity of 4-chloroimidazole against Gram positive and Gram negative bacteria

Bacteria	Activity	Diameter of Zones of inhibition (mm)
Gram positive bacteria		
Bacillus subtilis (NCIM 2063)	-	-
Staphylococcus aureus (NCIM 2079)	-	-
Gram negative bacteria		
Escherichia coli (NCIM 5010)	+	8
Psedumonas aeruginosa (NCIM 2862)	+++	30

Ciprofloxacin (10µg/ml) was used as positive reference standard antibiotic. -No inhibition, +, ++, +++, are zone diameter for 1-10mm, 10-20mm and higher than 20mm

respectively. pH =7.4, Temperature = 37.0 ^{0}C, Conc. 256 µg/ml

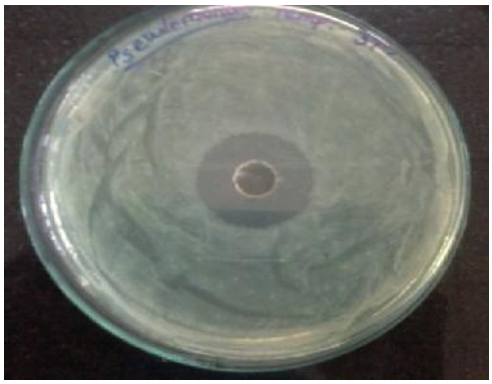


Figure 4: Inhibition zone caused by 4-chloroimidazole against *Pseudomonas aeruginosa* (NCIM 2862) at 37.0 ^{o}C, 7.4 pH, and *at* concentration 256µg/ml

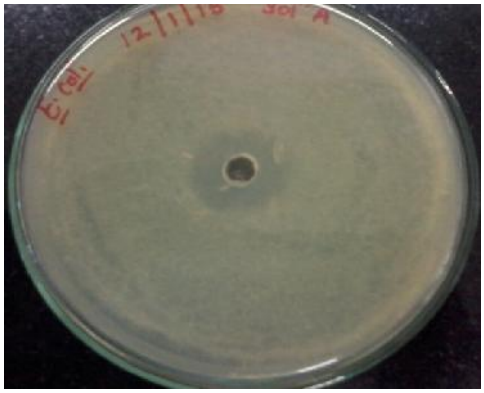


Figure 5: Inhibition zone caused by 4-chloroimidazole against Escherichia coli (NCIM 5010) at 37.0 ^{0}C, 7.4 pH and at concentration 256µg/ml

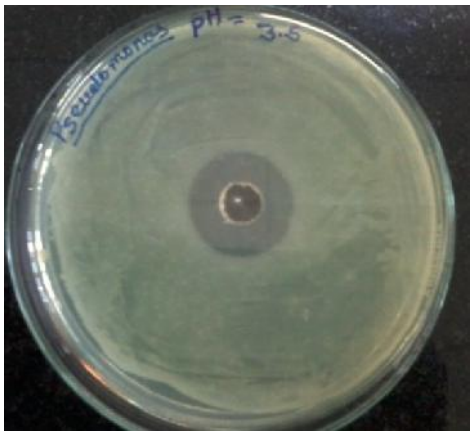


Figure 6: Inhibition zone caused by 4-chloroimidazole against *Pseudomonas aeruginosa* (NCIM 2862) *at pH = 3.5*

Table 4: Antibacterial activity of 4-chloro-imidazole against *Pseudomonas aeruginosa* at various pH

Bacteria	**pH of media**	**Diameter of Zones of inhibition (mm)**
Psedumonas aeruginosa (NCIM 2862)	3.5	23
Psedumonas aeruginosa (NCIM 2862)	5.0	25
Psedumonas aeruginosa (NCIM 2862)	7.4	30
Psedumonas aeruginosa (NCIM 2862)	8.0	26
Psedumonas aeruginosa (NCIM 2862)	10.0	24

Temperature = 37.0 ^{0}C, Conc. 256 «g/ml

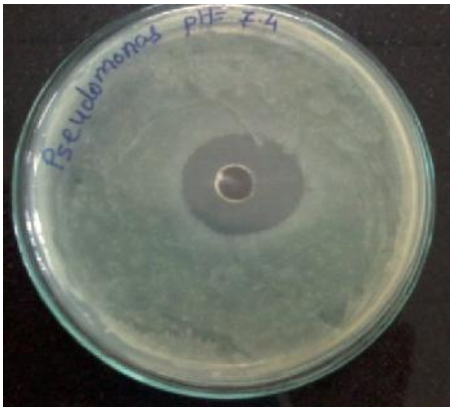


Figure 7: Inhibition zone caused by 4-chloroimidazole against *Psedumonas aeruginosa* (NCIM 2862) *at pH = 7.4*

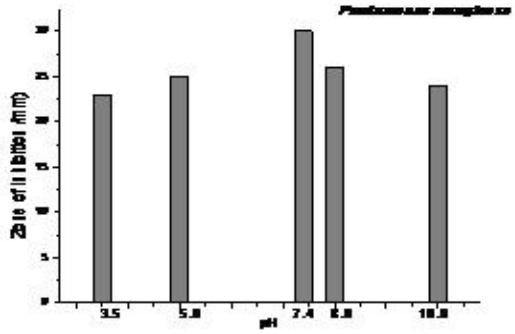


Figure 8: Comparative antibacterial activity of 4-chloroimidazole against *Psedumonas aeruginosa* (NCIM 2862) at various pH

Table 5: Antibacterial activity of 4-chloroimidazole against *Pseudomonas aeruginosa* at various temperatures

Bacteria	**Temperature (^{o}C)**	**Diameter of Zones of inhibition (mm)**
Psedumonas aeruginosa (NCIM 2862)	8.0	28
Psedumonas aeruginosa (NCIM 2862)	22.0	24
Psedumonas aeruginosa (NCIM 2862)	37.0	30
Psedumonas aeruginosa (NCIM 2862)	45.0	26

pH =7.4, Conc. 256 «g/ml

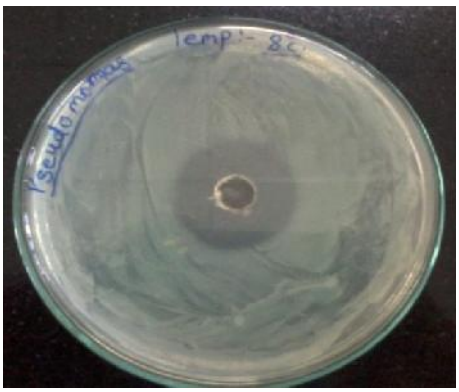


Figure 9: Inhibition zone caused by 4-chloroimidazole against *Psedumonas aeruginosa* (NCIM 2862) at 8.0^{o}C

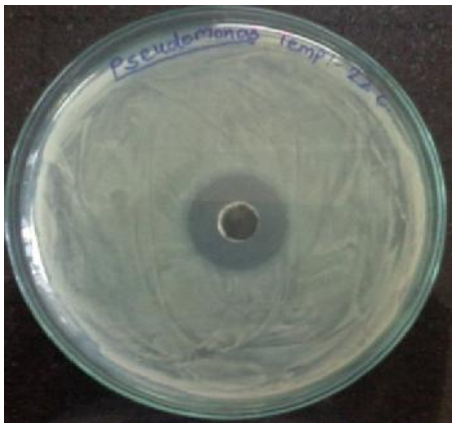


Figure 10: Inhibition zone caused by 4-chloroimidazole against *Psedumonas aeruginosa* (NCIM 2862) at 22.0^{o}C

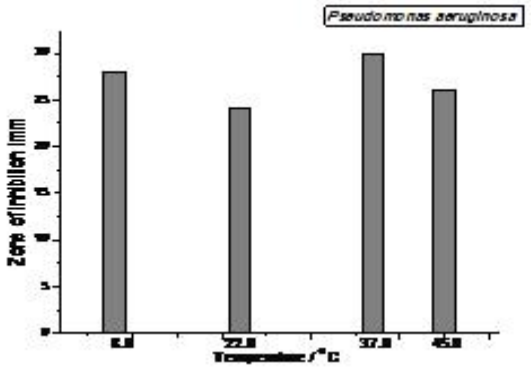


Figure 11: Comparative antibacterial activity of 4-chloroimidazole against *Psedumonas aeruginosa* (NCIM 2862) at various temperatures

Table 6: Antibacterial activity of 4-chloroimidazole against *Pseudomonas aeruginosa* at various concentrations

Bacteria	Conc. («g ml^{-1})	Diameters of Zones of inhibition (mm)
Psedumonas aeruginosa (NCIM 2862)	16	02
Psedumonas aeruginosa (NCIM 2862)	32	09
Psedumonas aeruginosa (NCIM 2862)	64	12
Psedumonas aeruginosa (NCIM 2862)	128	15
Psedumonas aeruginosa (NCIM 2862)	256	30

pH =7.4, Temperature = 37.0 ^{0}C

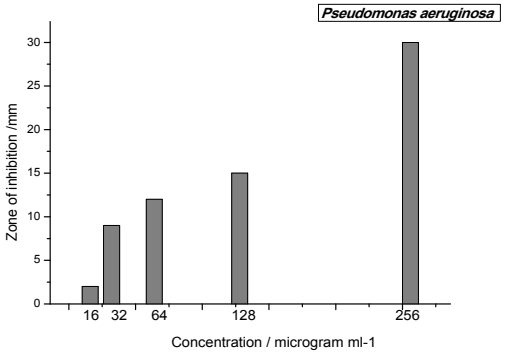


Figure 12: Comparative antibacterial activity against *Pseudomonas aeruginosa* (NCIM 2862) at various concentration of 4-chloroimidazole

Antifungal susceptibility assay

The product 4-chloroimidazole obtained from this kinetic study was also screened for the antifungal study against the various fungi and yeasts the results are tabulated in Table 7. The results shows that the compound exhibits good antifungal activity against all fungi under study namely *Aspergillus niger* (NCIM 545), *Penicillium notatum* (NCIM 741), *Penicillium chrysogenum* (NCIM 709), *Aspergillus fumigates* (NCIM 902), and *Penicillium chrysogenum* (NCIM 723). The product shows the high antifungal activity against the species such as *Penicillium chrysogenum* (NCIM 723), whereas moderate activity against the fungi such as *Penicillium notatum* (NCIM 741), *Aspergillus fumigates* (NCIM 902) and *Penicillium chrysogenum* (NCIM 09) and comparatively low activity against fungi *Aspergillus niger* (NCIM 545). The product was also screened for its activity against yeasts namely *Candida albicans* (NCIM 3100) and *Saccharomyces cerevisiae* (NCIM 3176). It is observed that the product fails to exhibit the anti yeast activity against *Candida albicans* (NCIM 3100) and less anti yeast activity against *Saccharomyces cerevisiae* (NCIM 3176).

Table 7: Antifungal activity of 4-chloroimidazole against test organism

S.No	Organism	Activity	Diameter of Zones of inhibition (mm)
	Fungi		
a.	*Aspergillus niger* (NCIM 545)	++	28
b.	*Penicillium notatum* (NCIM 741)	++	36
c.	*Aspergillus fumigates* (NCIM 902)	++	37
d.	*Penicillium chrysogenum* 1	++	39

	(NCIM 709)		
e.	*Penicillium chrysogenum* 2 (NCIM 723)	+++	53
	Yeast		
f.	*Candida albians* (NCIM 3100)	-	00
g.	*Saccharomyces cerevisiae* (NCIM 3176)	+	09

Ketoconazole (10 μg/ml) was used as positive reference standard antibiotic. - No inhibition, +, ++, +++, are zone diameter for 1-20mm, 20-40mm and higher than 40mm respectively

Figure 13: Inhibition zone caused by 4-chloroimidazole against *Aspergillus niger* (NCIM 545)

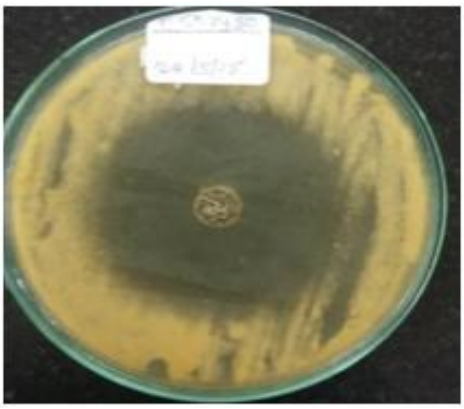


Figure 14: Inhibition zone caused by 4-chloroimidazole against *Penicillium chrysogenum* 2 (NCIM723)

The results of the antimicrobial screening showed that the compound 4-chloroimidazole posses the good antibacterial and antifungal activity. The higher antifungal activity observed in the present study for the compound 4-chloroimidazole compared to antibacterial activity. Moreover from perusal of the results, it was evident that the substituted imidazole may be used as templates to generate better drugs to fight against bacterial and fungal infections. Use of the imidazole derivative in recent drug development may show better effect and less toxicity.

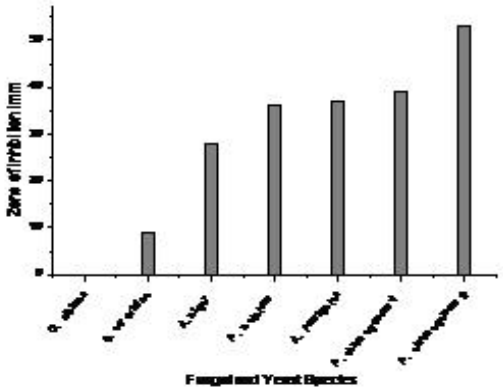


Figure 15: Antifungal acivity of 4-chloroimidazole against different fungi and yeast species

Mechanism of action

Imidazole derivatives are from the azole category. The azole antimicrobial agents prevent the synthesis of ergosterol, a major component of fungal plasma membranes, by inhibiting the cytochrome P-450 dependent enzyme lanosterol demethylase. Exposure of fungi to azoles causes the depletion of ergosterol and accumulation of 14α-methylated sterols. This interferes with the bulk functions of ergosterol in fungal membranes and disrupts the structure of the membrane and its functions. The net effect is to inhibit the fungal growth [28-30].

4. Conclusion

The easy and accessible procedure is used to obtain the product 4-chloroimidazole. The kinetic study of chlorination of imidazole shows that the reaction is very rapid and dilute solutions are required. The 4-chloroimidazole obtained from the kinetic study has screened for antimicrobial activity against the various bacteria, fungi and yeast species by agar well diffusion method. The product exhibits the inhibitory activity against several bacteria, fungi and yeast. It shows the strong inhibitory effect towards the fungi compared to bacteria and yeast. The present study may give the guidelines for design and optimization of new agent in recent drug development.

Table 2: Kinetics of chlorination of imidazole by N-chlorosucinimide in aqueous medium

Time (sec)	Diffusion Current (nA)	[NCS] (10^{-3} M)	$[NCS]^{-1}$ ($10^{2}M^{-1}$)
0	35.0	2.50	4.00
10	30.2	2.14	4.67
20	27.1	1.92	5.19
30	22.3	1.75	5.68
40	21.2	1.51	6.62
50	19.0	1.36	7.34

60	17.7	1.26	7.93
70	16.8	1.19	8.36

Initial concentration of NCS : 2.5×10^{-3} M
Initial concentration of substrates : 2.5×10^{-3} M
Concentration of KNO_3 : 0.25 M
Temperature : 301.6 K

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6. References

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