Chapter 4

Impact of dimers of 2-substituted-benzo[d][1,3]oxazin-4-ones on Antibacterial activity

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4.1 Introduction

In the preceding chapter, the existence of dimers in solution state was discussed. In the present chapter, the effect of the dimers on antibacterial activities is explored. The importance of such study lay in the fact that most of the drug molecules are capable of forming self association, while the effect of self association on drug action is not properly investigated. However, the dimerization of semisynthetic eremomycin derivatives and their effect on antibacterial activity was studied by Mirgorodskaya *et al* (2000)**.** Many classes of cytotoxic agents that have the potential to become effective anticancer drugs are hydrophobic, a property that is typically associated with poor solubility in aqueous medium (Khan *et al.,* 2006). The limited water solubility of hydrophobic compounds results from their propensities to form self-association. The key factor toward achieving highest activity is bioavailability, a factor that is likely to be impacted by self-association properties of the drug. Hence, it was important to understand compound's self-association/dimerization behaviour in solution and examine how this behaviour correlates with antibacterial activity.

4.2 Materials and methods

4.2.1 Reagents and Chemicals

All the chemicals and reagents used in the experiment were of analytical grade (AR) and purchased from E. Merck, India. The growth media or its components used were purchased from HiMedia, India.

4.2.2 Maintenance of bacterial strain

For short-term preservation and routine use, stock bacterial culture were maintained on Nutrient agar (NA) slant containing peptone,10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l; and agar 15g/l, at pH 7.0. LB agar medium was sterilized by autoclaving at 121 °C for 20 minutes and 4 ml was transferred into the test tubes to form LB agar slant. A loopful of bacterial cultures from the stock samples was streaked on LB agar slant and incubated at 37 °C for 24 h. The cultures were stored in the refrigerator at 4 °C. For long-term preservation, cultures in 10-15% glycerol were stored at -20 °C and sub-cultured after every 3 months.

Bacterial strains and growth experiments

All experiments were done using the strains *Escherichia coli K12, Bacillus subtilis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* 2257. Fresh inoculum was prepared by transferring a single colony of 24 h old cultures into 10 ml sterile nutrient broth (pH 7.0) in 100 ml Erlenmeyer flask. The inoculated medium was incubated at 37°C for 4 h with agitation. The culture was harvested by centrifuging at 8000 rpm for 5 min at 4 °C and washed twice with sterile phosphate buffer saline (PBS) to remove traces of media if any. The washed pellet was finally suspended in 3 ml sterile PBS. Aliquots of approximately 10^4 cells were added to 10 ml of nutrient broth in Erlenmeyer flask. The flask was kept at 37°C (with shaking at 200 rpm) throughout the period of investigation. Survivability of cells in LB was assessed through dilution-plating of pure culture aliquots at different time intervals on fresh LB agar plates. The number of cells present on that particular colony was also enumerated using dilution-plating technique.

4.2.3 Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activity was assayed against different bacteria *Escherichia coli K12, Bacillus subtilis, Staphylococcus aureus, and Pseudomonas aeruginosa* 2257 by turbidometric method. The test compound was dissolved in DMSO (SRL Extra pure) to prepare the stock solution and aseptically used for experiments. The required volume of

stock solution was transferred to tubes containing a defined volume of nutrient broth to achieve a desired concentration of the compound. The concentrations of the tested compound were 100, 400, 600, 800 and 1000 µg/ml in comparison to the standard drug ampicillin. Control set was contained DMSO, respective of each experimental tubes. A loopfull of bacterial culture from 24 h old slant was transferred to 10 ml of nutrient broth (Himedia M502) and incubated at 37 ^{0}C for 4 h. The tubes in duplicate containing 5ml nutrient broth were inoculated with 0.1 ml of 4 h liquid culture. The tubes containing nutrient broth were incubated at 37 ^{0}C for 16 h and the relative growths in the tubes were determined turbidometrically in the spectrophotometer. Taking the growth in tubes without drug as 100% (X), the percent growth (Y) in presence of studied compounds at particular concentration was calculated by the formula: (OD_{540} of the tube with drug / OD_{540} of the tube without drug)* 100. The inhibition % was calculated as X-Y (Nanda *et al.,* 2007).

4.2.4 Growth curve and Growth Kinetic study of *E. coli* K12 in presence of 2-substituted-benzo[d][1,3]oxazin-4-ones

The overnight culture of *E. coli* K12 (in NB medium), that were inoculated with a freshly grown single colony, was diluted 100-fold into 10 ml NB medium and allowed to grow for 4h to obtain log phase cells. The culture was then inoculated into 1000 ml NB medium and divided into five portions (C1, E1, E2, E3 and E4). Further each portion was distributed in four separate sterilized Erlenmeyer flasks and labelled as 100, 400, 600 and 800 (numerical represented their concentrations in µg/ml). Then to each labelled flask appropriate volume of compound was added from stock solution to achieve the desired concentration, while in C1 portion flasks only respective volume of DMSO was added (control). All Erlenmeyer flasks were incubated at 37^{o}C with shaking. Growth of *E. coli* K12 was quantified in terms of optical density at different time interval. (Miller, 1972). Culture was prepared as mentioned above. At different time intervals, cultures were withdrawn from time-defined flasks (both control and test) for measuring optical density. The growth rate constant was determined in between 4 to 6 hours in each case.

4.2.5 Mathematical evaluation of Dimerization on antibacterial activity

In order to separate the activity of the monomer and dimer we used the following equations: 2M=D, $K=[D]/[M]^2$; M+S = MS, K'=[MS]/[M]*[S]; D+S=DS, K''=[DS]/[D]*[S]; I=p*[M]+q*[D]; C=[M]+[MS]+2[D]+2[DS]; where M, D and S are monomer, dimer and bacterial substrate respectively and K, K' and K'' are corresponding association constants; I is the observed bacterial growth inhibition per mole, p and q are the corresponding contributions by the monomer and dimer respectively and C is the concentration in terms of monomer. With the consideration that the mole fraction of the bacterial drug absorbing surface is supposed to be one, hence [S] is considered as unity in the equilibrium equation, [S]≈1. The mathematical model used for evaluation of bacterial inhibition for the monomer and dimer separately:

$$M + \mathrm{M} = \mathrm{D} \quad (1)$$

$$\mathrm{K} = \frac{[\mathrm{D}]}{[\mathrm{M}]^2} \quad (2)$$

$$M + \mathrm{S} = \mathrm{MS} \quad (3)$$

$$\mathrm{K}' = \frac{[\mathrm{MS}]}{[\mathrm{M}][\mathrm{S}]} \quad (4)$$

$$[\mathrm{D}] + [\mathrm{S}] = [\mathrm{DS}] \quad (5)$$

$$K'' = \frac{[\mathrm{DS}]}{[\mathrm{D}][\mathrm{S}]} \quad (6)$$

$$\mathrm{I} = \mathrm{p} * [\mathrm{M}] + \mathrm{q} * [\mathrm{D}] \quad (7)$$

$$\mathrm{C} = [\mathrm{M}] + [\mathrm{MS}] + 2[\mathrm{D}] + 2[\mathrm{DS}] \quad (8)$$

$$\mathrm{C} = [\mathrm{M}] + \mathrm{K}'[\mathrm{M}] + 2[\mathrm{D}] + 2\mathrm{K}''[\mathrm{D}]$$

$$\mathrm{C} = (1 + \mathrm{K}')[\mathrm{M}] + 2(1 + \mathrm{K}'')[\mathrm{D}]$$

$$= (1 + K')[M] + 2K(1 + K'')[M]^2$$

$$[\mathrm{S}] \approx 1 \text{(Considered)}$$

$$2K(1+K'')[M]^2 + (1+K')[M] - C = 0 \qquad (9)$$

By putting, $m = (1+K'')$ and $s = (1+K')$

The eqn 9 turns to, $2Km[M]^2 + s[M] - C = 0$

$$[M] = \frac{-s+\sqrt{s^2+8KmC}}{4Km} \qquad (10)$$

Substituting [M] in equation (7) we get -

$$I = \left[\left\{p + q.K\left(\frac{-s+\sqrt{s^2+8KmC}}{4Km}\right)\right\}.\left(\frac{-s+\sqrt{s^2+8KmC}}{4Km}\right)\right]$$

4.3 Results and Discussion

4.3.1 Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activities of the synthesized compounds were evaluated against four bacteria, *E. coli* K12, *Pseudomonas aeruginosa* 2257, *Staphylococcus aureus* and *Bacillus subtilis*. MIC value of each synthesized compounds against bacterial culture is given in Table 4.1. The obtained MIC of 3-aryl-deneamino-2-phenyl-quinazoline-4-(3H)-one was matched with the previous report. But, to assist interpretation of sensitivity of compounds 2-substituted-benzo[d][1,3]-oxazine-4-one (1a-1d), an in-vitro breakpoint *i.e.* > 1000 µg/ml for these compounds were assigned. From this data MIC breakpoint of > 1000 µg/ml has found for these compounds only against *E. coli* K12, however they did not show any inhibitory effect against other three bacteria.

4.3.2 Growth kinetics of *E. coli* K12 in the presence of compound 1a-1d

As it was intended to explore the effect of the dimers on antibacterial activities, growth kinetics was studied at different concentration of 2-substituted benzo[d][1,3]-oxazine-4-one against *E. coli* K12. The increase in OD at 540 nm, which represents growth of *E. coli* K12, was plotted against time at different concentrations (Figure 4.1).

Table 4.1 Antibacterial activity (MIC) of synthesized compounds 1a-1d, 2a and 3a-j.

Compound	**R**	**Gram-negative**		**Gram-positive**	
		E. coli K12 (µg/ml)	*P. aeruginosa* 2257(µg/ml)	*S. aureus* (µg/ml)	*B. subtilis* (µg/ml)
1a	-	> 1000	ND	ND	ND
1b	-	> 1000	ND	ND	ND
1c	-	> 1000	ND	ND	ND
1d	-	> 1000	ND	ND	ND
2a	-	400	800	ND	400
3a	2′′-OH	200	300	200	300
3b	4′′-OCH_3	200	200	200	100
3c	4′′-F	200	200	100	200
3d	4′′-$N(CH_3)_2$	100	200	100	100
3e	4′′-Cl	200	300	100	100
3f	3′′-OCH_3	400	200	200	100
3g	4’-OH	400	300	200	300
3h	3′′-OCH_3, 4′′-OH	100	500	200	200
3i	3′′-NO_2	200	200	300	200
3j	H	200	500	100	100

ND- Not Defined

The corresponding growth rate constants (µ) were determined within time interval of 4-6 hours (Figure 4.2). The results revealed that growth and growth rate constants decreased with increasing concentrations of the compound(s). The results revealed that 1a was the only compound which showed significant inhibition against *E. coli* K12. Lysis of bacterial cells is observed in the death-phase. The phase contrast microscopic image shows some morphological changes suggestive of distortion of bacterial cell as well. So, we have used *E. coli* K12 as model organism for further study.

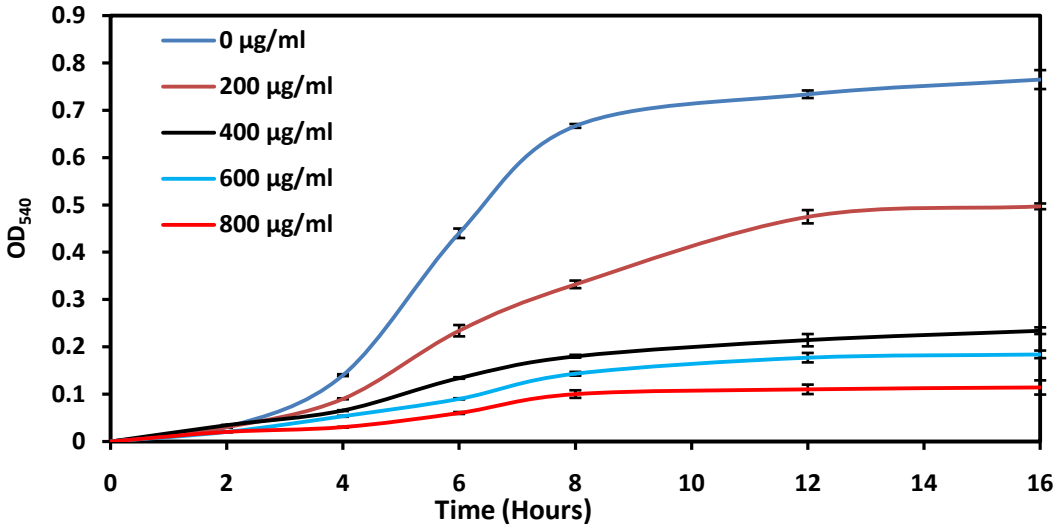

Figure 4.1(a): Growth curve of *E. coli* K12 in presence of 2-phenyl benzo[d] [1, 3] oxazin-4-one **(**1a).

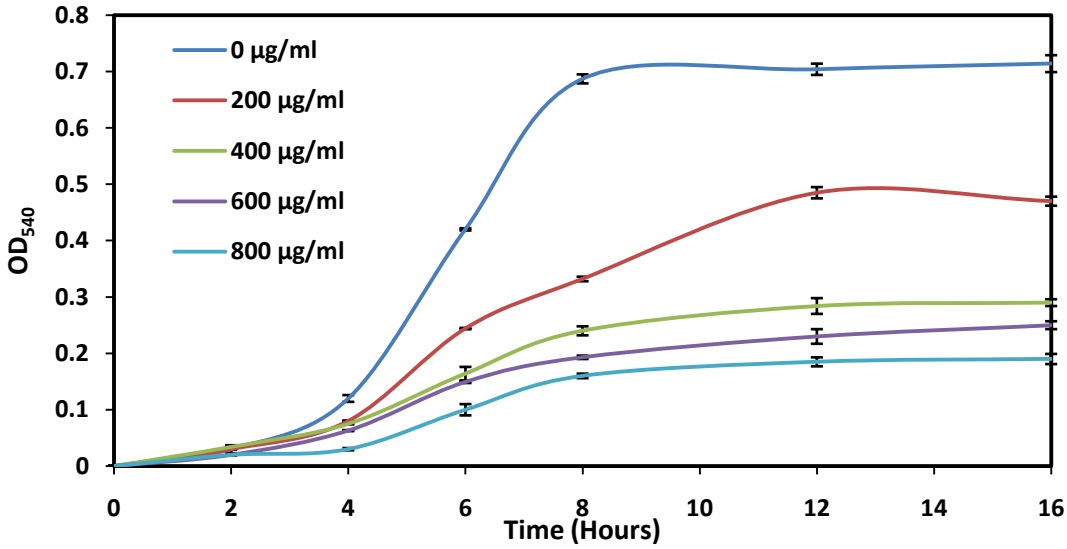

Figure 4.1(b): Growth curve of *E. coli* K12 in presence of 2-p-tolyl-benzo[d] [1, 3] oxazin-4-one **(**1b).

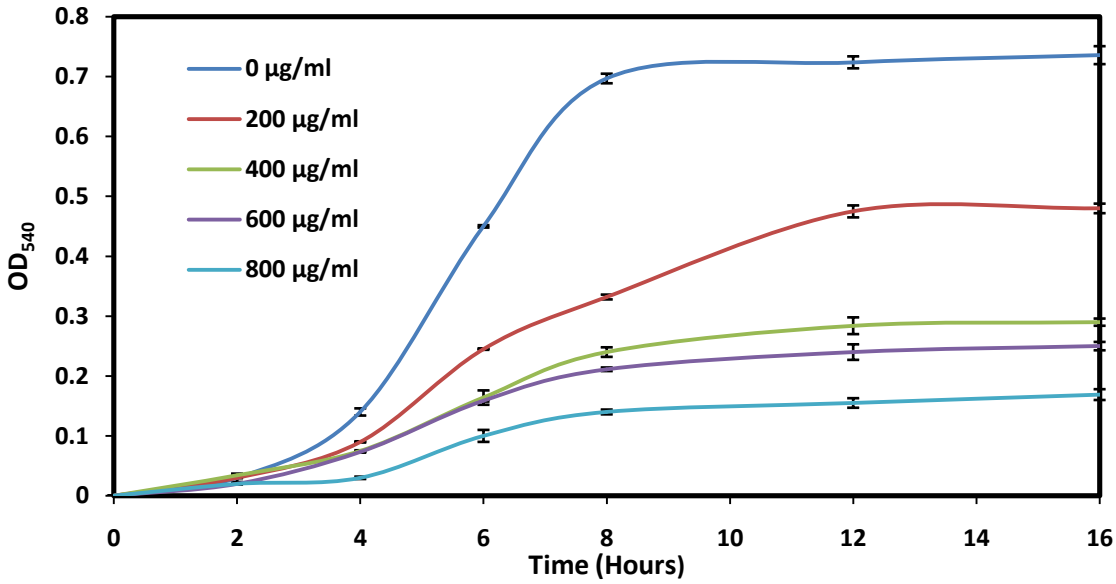

Figure 4.1(c): Growth curve of *E. coli* K12 in presence of 2-p-chlorophenyl-benzo[d] [1, 3] oxazin-4-one (1c).

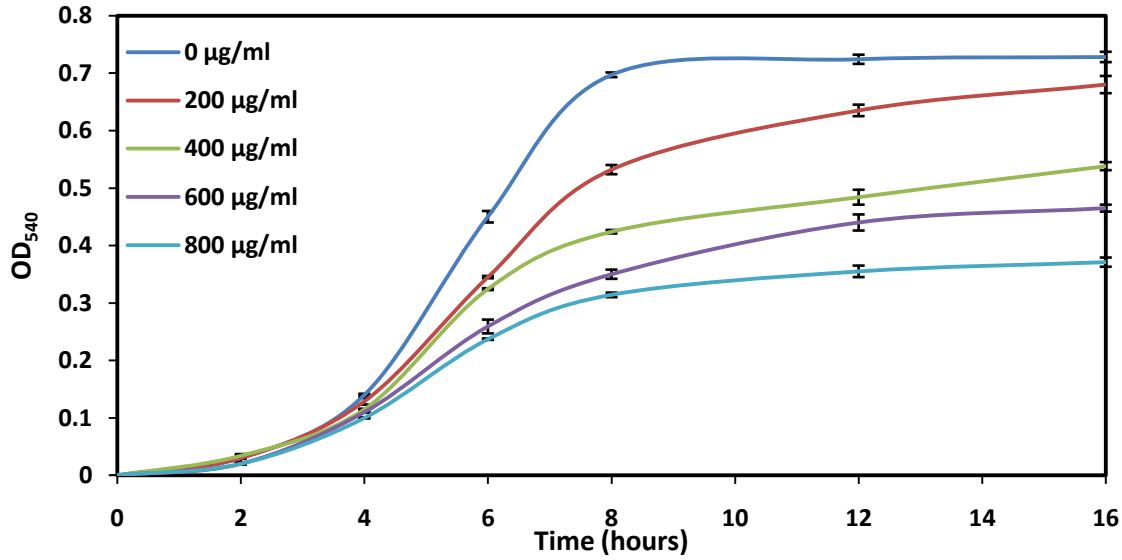

Figure 4.1(d): Growth curve of *E.coli* K12 in presence of 2-p-chlorophenyl-benzo[d] [1, 3] oxazin-4-one (1d).

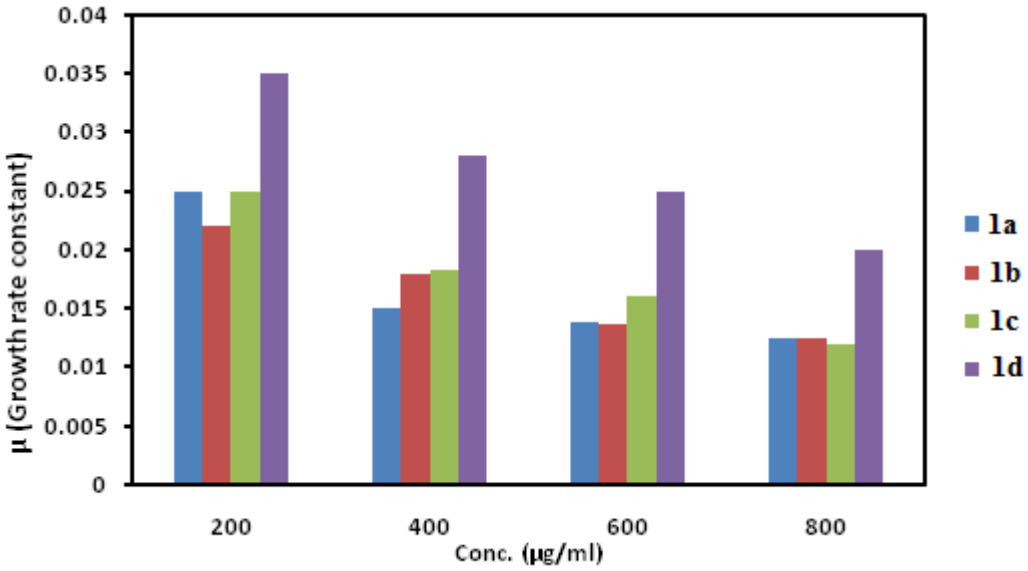

Figure 4.2: Representation of Growth rate constants of *E. coli* K12 in presence of compounds 1a, 1b, 1c and 1d

4.3.3 Impact of Dimerisation on Bacterial Inhibition

To study the effect of dimerization on antibacterial activity, the antibacterial activities of the compounds were assayed against the gram negative bacteria *E. coli* K12. Figure 4.3 shows the percent inhibition (percent of inhibition respective to control) of bacterial growth in presence of studied compounds at different concentrations.

It is apparent from the plot that the change of percent inhibition of the compound 1d at different concentration is minimal, while compound 1a and 1c have the higher values. The percent inhibition values used in this graph when converted to molar inhibition *i.e.* percent inhibition per mole of compound, as obtained by dividing the percent inhibition with the respective mole of compounds in 100, 400, 600 and 800 µg/ml to corresponding molar amount.

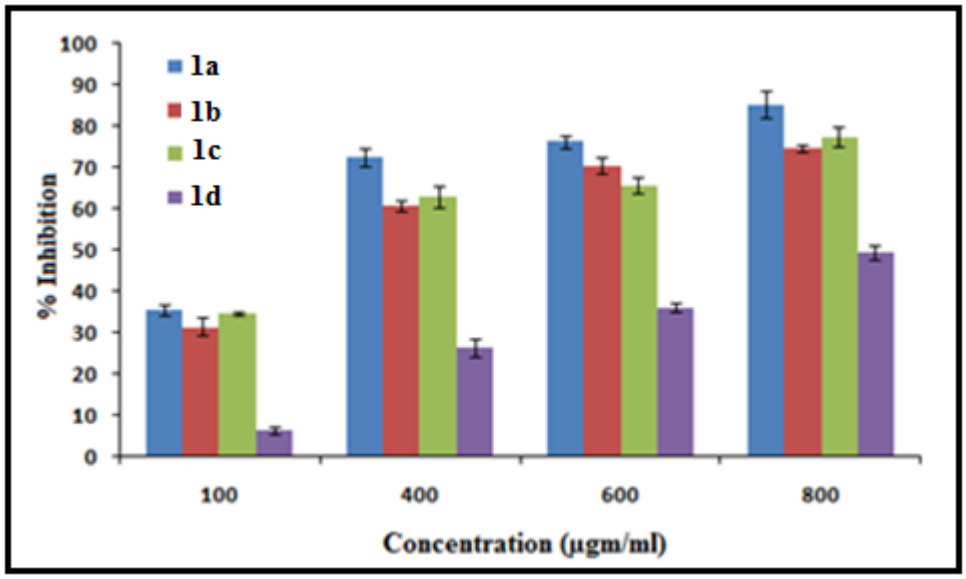

Figure 4.3: Percent Inhibition of bacterial growth against *E. coli* K 12 at different concentrations of compounds (1a-1d).

This chart is interesting because it tells that the compounds' potentiality to antibacterial effect is markedly higher at dilute solution and gradually decreased at higher concentrations. Though when expressed in micrograms the percent inhibition it is found to increase with the increase of the amount of the said compounds (Figure 4.4).

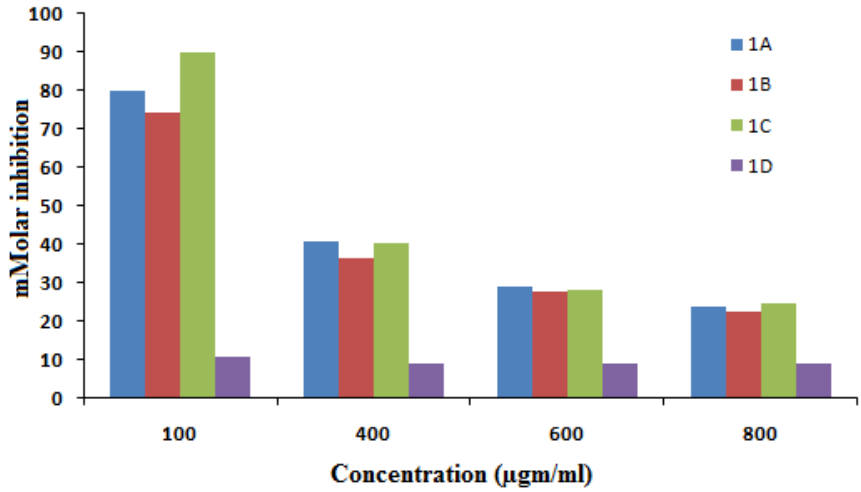

Figure 4.4: mMolar inhibition of bacterial growth (*E. coli* K12) at different concentrations of compound 1a - 1d.

To the best of our knowledge no other studies have expressed percent inhibition in molar terms. But we find it convenient to judge the antibacterial property at different concentrations in molar term. This term clearly states that the property per mole in different set of concentrations.

The apparently new phenomenon is similar to monomer dimer equilibrium in solution, where percent of monomer increases with dilution. Similar pattern was also observed in previous studies. Similar to the present study, earlier authors have found more monomer of antitumour agent novatrone (mitoxantrone) drug in higher dilution (Davies *et al.,* 2000). In fact this observation forced us to the search of monomer dimer equilibrium in solution for the studied compounds. For the sake of rational arrangement of the chapters we described such studies in the earlier part of the thesis.

The observed molar inhibition values are fitted in the derived equation at different concentrations. A good fit was observed in all cases.

Table 4.2: Association constants of monomer and dimer with bacteria (per molar) and coefficients p and q.

Compound	*p*	*q*	*k*	*m*	*s*
1a	1.4E+06	9.1E+03	0.24	1.03	9.4
1b	7.8E+05	0.00013	0.14	1.8	5.8
1c	1.7E+07	0.001	0.17	1.5	10.7
1d	1E+06	15.9	0.21	1.2	1.03

The contribution of monomer to the antibacterial activity, p, is always much higher than that of the dimer, q. These results are the clear mathematical indication that the monomer is mainly responsible for the antibacterial activity. And the expected preponderance of monomer proportion at dilute solution is responsible for the observed higher molar activity at dilute solutions. The electron withdrawing chlorine atom at 4' position in 2-phenyl-ring increased the antibacterial property and electron donating methyl group at this position reduced the property of the monomer.

The dimer activity indicted from the values of q, is negligible but non zero. The monomer – dimer equilibrium constant values (k X 10^4) in bacterial medium are expectedly low in the bacterial medium than that in water. The monomers bind better than the corresponding dimers as ″s″ values are found to be higher than the corresponding ″m″ values (Table 4.2). The nearly flat nature of the molar inhibition property (Figure 4.4) of the compound 1d can now be safely assigned to be due to the monomer buffering effect originating from the existence of two metamorphic dimers in solution state. The curve fit graph represented that the experimental inhibition has also matched with our observed inhibition percentage (Figure 4.5).

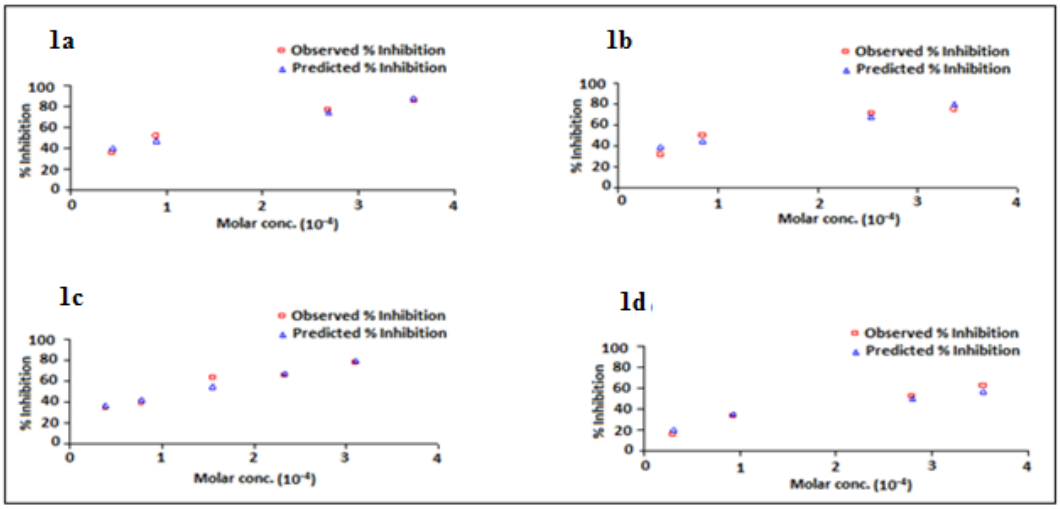

Figure 4.5: Curve fit plot of inhibition percentage against molar concentration of compound 1a, 1b, 1c and 1d.

We have pointed out that there is some intrinsic predicament for achieving much better antibacterial activity (Nanda *et al.*, 2007) of the molecules having this type of pharmacophore. Therefore, in the present study we have tried to address some reasons for that. No a priori work indicted the effect of self association of the drug molecules in their biological activity. In this sense, our method of SAR study is totally new of its kind.