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An Imperative Study on the Toxicological Evaluation of Three Antibiotics

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Abstract: **Antibiotics are highly powerful bioactive compounds which annul or restrict the growth of microorganisms. The unrestricted use of antibiotics has resulted in their emergence in the environment. Today, the whole of the world has acknowledged their presence in different natural and artificial systems. Surface waters (lakes, rivers, streams and sea), ground water, drinking water, soil, sediment and sludge have been reported for contamination with antibiotics. In the environment non-target microorganisms are inexorably exposed to antibiotics resulting in a potential risk of negative effects on indigenous microorganisms. Thus it is essential to monitor and assess the impact of antibiotics in the environment especially on microorganisms present. Therefore, this paper attempts toxicological characterization of antibiotics in order to investigate their probable toxic effects on microbial population. A bacterial short term bioassay viz. *Pseudomonas fluorescens* growth inhibition assay was used to determine the toxicological effects of three commonly used antibiotics procured from pharmacy stores located in Jaipur, Rajasthan (India).**

Keywords: **antibiotics, toxicity, growth inhibition, pollution, microbial assays.**

1. INTRODUCTION

Rapid innovations in the health-care sector have resulted in the development of antibiotics. Ever since it has been realized that antibiotics can be used to treat and prevent infections, their market has been expanding out of bounds. They are extensively and effectively used in human and veterinary medicines and their benefits have also been realized in agriculture, aquaculture and in livestock as growth promoters. However, with the increase in the production of antibiotics began the entering of their effluents into the environment.

Antibiotics are not metabolized completely by our body when we take them for medicinal purposes. Some are expelled as waste and enter into wastewater treatment plants (WWTPs) where even the best tertiary treatment is unable to disable antibiotic activity [17]. This wastewater is then released into waterways where it gets transferred to large areas. Several sources of antibiotics have been identified such as wastes of pharmaceutical manufacturing plants [2, 11], disposal of unused and expired medicine [3] and landfill leachates [7]. Hence, antibiotics enter the environment through two main routes: urban and agricultural.

In the urban route, antibiotics excreted or discarded by people in households, hospitals or industries end up in sewers. Once in wastewater, antibiotics are either discharged directly into surface water or transported by sewers to wastewater treatment plants (WWTPs). Since conventional treatment plants cannot remove antibiotics from the wastewater, they are considered as the main entry point of urban antibiotics into the aquatic environment [6]. In agricultural route, antibiotics present in the animal excreta may reach the aquatic environment through surface run off and drainage or by percolation

into groundwater [9, 10]. Antibiotics can also reach natural waters directly from leaking manure storage structures or constructed lagoons [13]. Antibiotics used in aquaculture are directly released into surface waters by leaching through food pellets, fish faeces or pond sediments [4, 12]. Therefore, agricultural activities may be considered as the main non-point sources of antibiotics in the environment.

Once antibiotics enter the environment, they have the ability to change the structure of the microbial community. The reason is that antibiotics in general, even though they are designed to be broad-spectrum, have their selective effects on various groups of microbes. As a result, the selective effects of antibiotics alter the relative abundance of microbial species and consequently interfere with the various interactions among different species such as nutrient cycling, organic matter mineralization and degradation of pollutants [1]. Therefore, it is important to monitor and assess the impact of antibiotics on non-target organisms present in the environment.

Thus, concerning the possible threat generated due to unbridled use of antibiotics, this paper attempts toxicological characterization of antibiotics in order to investigate their probable toxic effects on microbial population. A bacterial short term bioassay *viz. Pseudomonas fluorescens* growth inhibition assay was used to determine the toxicological effects of three selected antibiotics which are commonly used in India.

2. MATERIALS AND METHOD

A. Sampling:

The sampling was done twice from a local pharmacy store of Jaipur city (Rajasthan, India). Following samples of antibiotics were collected during the two samplings:

1. Amoxicillin clavulanate

2. Cefuroxime

3. Roxythromycin

A 10 mg/L stock solution was prepared using sterile distilled water in all these samples. This solution was taken as 100% and further dilutions were prepared. Six dilutions (2%, 5%, 10%, 20%, 50% and 100%) of antibiotics were tested in duplicates in each experiment.

B. Bioassay:

Pseudomonas fluorescens growth inhibition assay:

The tester strain of *Pseudomonas fluorescens* (MTCC103) was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTEC), Chandigarh, India. The assay was carried out as described by Dutka and Kwan, (1981) [5]. In this assay, 15mL of *P. fluorescens* culture grown overnight was aseptically inoculated into 1L of sterile nutrient broth. Then 25mL of this test inoculum was dispensed into conical flasks. To each flask, 25 ml of antibiotic solution was added and then the flasks were placed in rotatory shaker at 30^{o}C for 10 hours. Different dilutions (2, 5, 10, 20, 50 and 100%) were analysed for each antibiotic to determine bacterial response to these dilutions. After 10 hours, the inhibition of *Pseudomonas* growth was evaluated by measuring the absorbance on a spectrophotometer at 650 nm. In this assay the criterion for growth inhibition is the reduction in cell multiplication determined as the reduction in growth of the culture. After proper incubation of the culture, growth was determined as turbidity during this period. Inhibition of an increase in turbidity or the % growth inhibition in the samples was compared with that of the negative control. All the chemicals and reagents used in the assay were of analytical grade, supplied by Hi-Media Laboratories Limited.

C. Data Analysis:

In the assay, reduction in the growth of the culture is determined by measuring the turbidity of the culture after a proper incubation period. Percent growth inhibition or inhibition of an increase in turbidity in the samples was compared with the negative control using the following equation:

$$\%I = OD_c - OD_t \,/\, OD_c \times 100$$

Where, %I is the growth inhibition, expressed in percentage, OD_t is the optical density (at 650nm) of a culture incubated with 'x' concentration of test sample and OD_c is the measured turbidity i.e. optical density of culture at the end of the test period in the negative control.

Concentrations causing 20% and 50% decrease in the number of cells are known as EC_{20} and EC_{50} respectively where EC stands for effective concentration. EC_{20} and EC_{50} values were calculated using probit method of logistic regression using MS-excel software XLSTAT and dose-response curves were drawn using MS-Excel 2007.

3. RESULTS AND DISCUSSION

There are very few or no studies regarding the hazardous effects of antibiotics on microorganisms present in the environment in India. Therefore, this study is amongst the first few initial studies in India attempting to measure and compare the cytotoxic potential of three antibiotics commonly used in India.

When the samples were analyzed with the *P. fluorescens* growth inhibition assay, responses observed were more or less similar in both the samplings (Figure 1 a & b). The observations revealed that amoxicillin clavulanate was least toxic out of the three antibiotics tested. The EC_{20} values obtained using XLSTAT software was 22.75% for sampling 1 and 49.95% for sampling 2. EC_{50} values for amoxicillin clavulanate could not be calculated for both the sampling as it failed to kill 50% cells even at 100% concentration (Table 1 & 2). On the other hand, roxythromycin was observed to be the most cytotoxic in this assay. During the first sampling, this sample gave an EC_{20} and EC_{50} value of 1.04% and 5.31% respectively. This indicated that half of the cells were killed at a low dose of 5.31%. For the second sampling, the value of EC_{20} and EC_{50} were 1.22% and 6.97% respectively indicating no significant change during both the samplings (Table 1 & 2). The third antibiotic, cefuroxime showed significant cytotoxicity in this assay. The EC_{20} values were 7.23% and 11.65% during the consecutive samplings while EC_{50} value was 19.47% during sampling 1 and 25.32% during sampling 2 as seen in Table 1 & 2. In all the three antibiotic samples growth inhibition increased with the increase in their concentration as observed in Figure 1 a & b.

The results obtained from this assay are significant because *P. fluorescens* is a non-target organism for all the three antibiotics used in the present study and despite that all three of them were able to show a certain degree of toxic effect on this organism indicating that antibiotics at low concentration may have the potential to harm non-target organisms present in the environment.

These above findings are in agreement with several other studies which indicate that antibiotics have the ability to cause lethal effects on non-target organisms [8, 14]. In 2000, Wollenberger and his co-workers studied toxicities of nine antibiotics on *Daphnia magna* (fresh water crustacean). It was found that antibiotics were toxic to *D. magna* at concentration as low as 4.6 mg/L [16]. Several other aquatic organisms have been used by researchers to study the effects of different antibiotics on them. One such example is when Robinson and his co-workers (2005) studied the toxicity of seven fluoroquinolones on five aquatic organisms viz. *Microcystis aeruginosa* (cyanobacteria), *Lemna minor* (duck weed), *Pseudokirchneriella subcapitata* (green algae), *Daphnia magna* (crustacean) and *Pimephales promelas* (fish). It was found that *M. aeruginosa* was the most sensitive followed *by L. Minor and P. subcapitata*. While *D. magna* and *P. promelas* showed limited toxicity [15]. All these findings are in support of the results obtained with the present study.

TABLE 1: EC_{20} and EC_{50} values for antibiotics in *Pseudomonas fluorescens* growth inhibition assay during sampling 1

S.No.	Samples	EC_{20}*		EC_{50}*	
		%	mL**	%	mL**
1	Amoxicillin clavulanate	22.75	5.69	-	-
2	Roxythromycin	1.04	0.26	5.31	1.33
3	Cefuroxime	7.23	1.81	19.47	4.87

*EC_{20} and EC_{50}: values of concentration (%) of sample showing 20% and 50% inhibition in the growth of *Pseudomonas* cells.

**Volume of antibiotic solution added to distilled water to make volume upto 25 mL.

'-' indicates high EC_{50} values (more than 100%)

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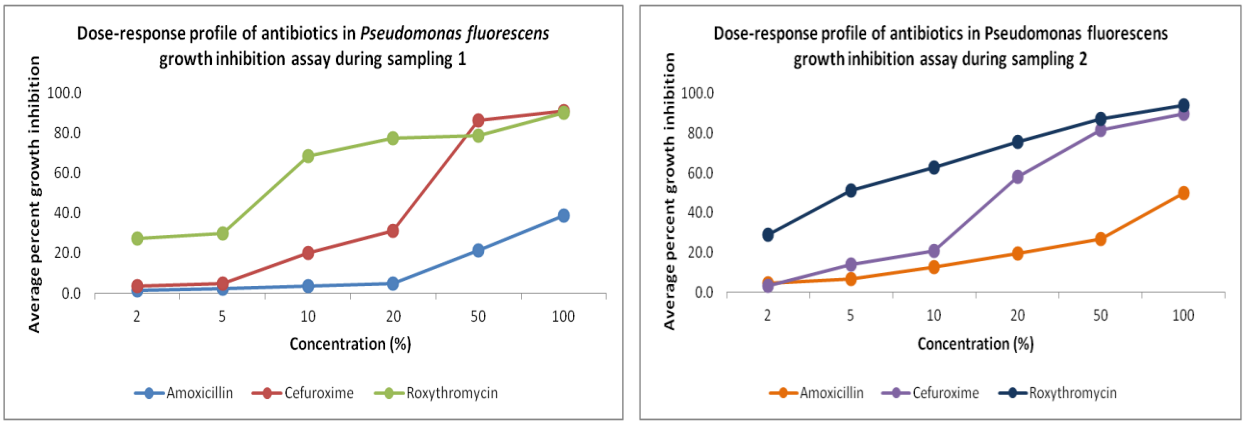
TABLE 2: EC_{20} and EC_{50} values for antibiotics in *Pseudomonas fluorescens* growth inhibition assay during sampling 2

S.No.	Samples	EC_{20}*		EC_{50}*	
		%	mL**	%	mL**
1	Amoxicillin clavulanate	49.95	12.49	-	
2	Roxythromycin	1.22	0.31	6.97	1.74
3	Cefuroxime	11.65	2.91	25.32	6.33

*EC_{20} and EC_{50}: values of concentration (%) of sample showing 20% and 50% inhibition in the growth of *Pseudomonas* cells.

**Volume of antibiotic solution added to distilled water to make volume upto 25 mL.

'-' indicates high EC_{50} values (more than 100%)



(a) **(b)**

FIGURE 1: Dose-response curves of antibiotics in *Pseudomonas fluorescens* growth inhibition assay (A) during sampling 1 and (B) during sampling 2.

4. CONCLUSION

In recent years, toxicity testing has grown steadily and is a useful tool in environmental risk assessment. Present studies which are available to determine the biological impacts of toxicants i.e. the impacts of chemical toxicants on living beings are often expensive, require large volume of samples and are time consuming. Due to all these disadvantages/limitations the present study deal with the use of rapid, reproducible and cost effective bacterial assay for toxicity screening and assessment of three antibiotics. Thus a short term microbial bioassay like *P. fluorescens* growth inhibition assay seems to be relevant for assessing the toxic potential and health hazard caused by antibiotics to aquatic and terrestrial microorganisms, upto a considerable extent. Furthermore, based on the observations with short term *in vitro* tests, it is recommended to also test these antibiotics in *in vivo* animal studies in order to determine the potential relevance of the toxic effects of antibiotics regarding potential health effects in humans and other higher aquatic and terrestrial organisms.

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