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Ecotoxicological Studies of Cephalosporin Antibiotics on *Daphnia Magna*

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Abstract

The present research was outlined to survey the intense and incessant poisonous quality of Cefixime and Cefotaxime antibiotics on *Daphnia magna*. The toxicity of Cefixime and Cefotaxime antibiotics was evaluated by 48h acute toxicity (US, EPA, 2002) and chronic toxicity (OECD guidelines 211 (OECD, 2012)). The data showed that EC_{50} of Cefixime was 78.78 mg/l and for Cefotaxime was 25.82 mg/l. *Daphnia magna* showed more sensitivity to Cefotaxime, when compared with Cefixime antibiotic. Chronic toxicity showed that, Cefotaxime cause effects on reproduction but Cefixime had no adverse effect on *Daphnia*. The mortality rate enhanced and average number of neonates diminished as exposure to Cefotaxime antibiotic was increased. *Daphnia* died (50%) at a concentration of 16.2 mg/l of Cefotaxime.

Keywords: Cephalosporin, Non Target Organisms, Reproduction, Toxicity, *Daphnia magna*

1. Introduction

Among the groups of pharmaceuticals, antibiotics are the most prescribed class of drugs[8] for the treatment of human diseases and as veterinary drugs and growth promotion[48,49]. Recent estimation of antibiotic used worldwide was between 100,000 and 200,000 tons/year[19,49]. These compounds undergo metabolic changes after consumption and the remaining metabolized forms are excreted via urine and feces and released into the aquatic environment[43,48]. Several researchers have reported the presence of antibiotics in different water compartments, including WWTP effluents[11,16,24] sediments[2,11,26,28] surface water[20], ground water[13,32], and seawater[46].

Presence of antibiotics in the environment can affect aquatic organisms. Studies have reported their effect on growth, body weight, survival of microflora and microfauna[4,42,45,48] allergic reactions, development of antibiotic-resistance and toxicity[7]. Studies on toxic effect of antibiotics have clearly stated that antibiotics are potential genotoxic agent which is proved through animal and microbial assays[10]. Various tests have been designed to assess the toxicity effects of antibiotics like SOS chromo test on *E. coli*, point mutation test (Ames test) on Salmonella species[22]. Higher plants also being used as the model to test the genotoxicity of antibiotic through micronucleus formation, root tip chromosomal aberration assay and sister chromatid exchange[27]. In the recent times, their effects on aquatic organisms and plant species have been studied under controlled laboratory conditions[5]. Cefixime and Cefotaxime belongs to class beta-lactams are broad spectrum antibiotics; one of the most widely used antimicrobials[33], and were dominant during previous 50 years[46]. They are considered to be the most prominent antibiotics for the treatment of human and livestock respiratory diseases, accounting for about 50-70 percent of the total antibiotics used in most countries[29].

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The present study evaluates the acute and chronic toxicity of Cephalosporin antibiotics on *Daphnia magna*. The model organism was chosen because of its importance in the freshwater environment, as it is on the important food chain between algae and fish[25]. Due to its life cycle, common distribution, high fertility and sensitivity to toxins and ease of handling in laboratories *Daphnia* has been used extensively in regulatory testing and ecotoxicological research[31].

2. Materials and Methods

2.1 Chemicals

Cefixime (CAS-79350-37-1) and Cefotaxime (CAS-64485-93-4) antibiotics were purchased from Yarrow chemicals (India); purity ≥ 98%. Standard stock solution of Cefixime and Cefotaxime antibiotics (500 mg/l) was prepared. Test solutions were prepared for each test species with the appropriate dilution medium. The solution was stored in a dry and dark place, at above 18^0C for no longer than 2 days before use.

2.2 Test Organism

Daphnia magna were maintained at temperature 20±1°C with photoperiod of 16L: 8D cycle according to the Organization for Economic Cooperation and Development (OECD) test guideline 211 (OECD, 2012)[27]. *Daphnia magna* was fed daily with yeast and algae, renewed water twice a week. Similar sized *Daphnia magna* (< 24 hours) were used for this study[31].

2.3 Acute Toxicity Test

The modified OECD standard procedure was used for the acute toxicity testing. The test concentration limit was selected based on range finding test. For each test concentrations (i.e. 0, 2.5, 8, 25 and 250 mg/l), 5 individuals of *Daphnia magna* were placed in a 100 ml glass beaker containing 50 ml solution. Throughout the tests no additional food added and test solution was also not renewed. The immobility of *Daphnia magna* was recorded at 48 hrs. Individuals which were unable to swim within 15s under gentle agitation were regarded as immobile. After 48 hours of exposure, the concentration of Cefixime and Cefotaxime antibiotics that cause 50% immobility (EC_{50}) was calculated. The tests were repeated thrice.

2.4 Chronic Exposure of *Daphnia magna*

The OECD test guideline 211 (OECD, 2012) was followed for chronic toxicity tests. Chronic exposure concentrations were selected based on 48 hrs EC_{50} from acute toxicity tests. The test animals (<24-h old neonates) were exposed to Cefixime (0, 0.70, 2.1, 6.3, 18.19, and 56.7 mg/l) and Cefotaxime (0, 0.20, 0.60, 1.8, 5.4 and 16.2 mg/l) using ten individual in the form of replicates, during 21 days. The chronic toxicity was based mainly on the reproduction test and the concentration of the effect was one magnitude below the acute toxic level. The parameters of water quality were measured before and after every renewal of the medium and the medium were changed daily. The endpoints analysed along the test were the survival (%), first day of brood, number of young per female, number of young per brood.

2.5 Statistical Analysis

The experiments have been repeated three times independently and the average values and Standard Deviation (SD) have been used. The value of 48 EC_{50} (immobility) was calculated using the probit method (US EPA Probit Analysis Program, Ver. Oh, 1.5). One way Analysis of Variance (ANOVA) with Tukey's multiple comparisons was carried out to detect significant differences between groups using the SPSS software (Ver. 15.0). A confidence level of 5% was considered as the least significant difference of means for all analyses.

3. Results

3.1 Acute Toxicity of *Cefixime* and *Cefotaxime* to *D. Magna*

The acute test was used to determine the concentration range for the reproduction test. To investigate the aquatic

Table 1. Acute toxicity test results of Cefixime antibiotic of *Daphnia magna*

Concentration →	Control		2.5 mg/l		8 mg/l		25 mg/l		80 mg/l		250mg/l	
Exposure time →	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
A (k/m)	5/0	4/1	5/0	5/0	5/0	5/0	4/1	2/3	4/1	3/2	3/2	1/4
B (k/m)	5/0	5/0	5/0	3/2	3/2	4/1	4/1	5/0	3/2	¼	3/2	2/3
C (k/m)	5/0	5/0	5/0	4/1	5/0	4/1	5/0	3/2	3/2	2/3	3/2	2/3
D (k/m)	5/0	5/0	5/0	5/0	5/0	4/1	2/3	5/0	2/3	3/2	2/3	3/2
% Mortality	0	5	0	15	10	15	25	25	40	55	45	70

A, B, C, D is four different experimental groups; k/m indicates number of alive (k) and dead (m) animals in each group.

Table 2. Acute toxicity test results of Cefotaxime antibiotic of *Daphnia magna*

Concentration	Control		2.5 mg/l		8 mg/l		25 mg/l		80 mg/l		250 mg/l	
Exposure time	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
A (k/m)	5/0	4/1	5/0	5/0	5/0	4/1	4/1	2/3	4/1	1/4	1/4	0/5
B (k/m)	5/0	5/0	5/0	3/2	3/2	4/1	4/1	2/3	3/2	1/4	1/4	2/3
C (k/m)	5/0	5/0	4/1	5/0	5/0	4/1	5/0	3/2	5/0	2/3	2/3	0/5
D (k/m)	5/0	5/0	5/0	4/1	5/0	4/1	3/2	3/2	2/3	2/3	3/2	0/5
% Mortality	0	5	5	15	10	20	20	50	30	70	65	90

A, B, C, D is four different experimental groups; k/m indicates number of alive (k) and dead (m) animals in each group.

toxicity of Cefixime (Table 1) and Cefotaxime (Table 2) antibiotics, the immobility of *Daphnia magna* under antibiotic exposure were examined. The results obtained in the acute toxicity tests, showing the calculated 48 hrs EC_{50} for the two antibiotic compounds tested.

A clear dose- response relationship could be observed (percentage of immobility organisms). The data obtained for the drugs tested showed significant variability with following relative toxicity ranking: the EC_{50} value found to be 77.9 mg/l and 28.82 mg/l for Cefixime and Cefotaxime respectively after 48 hrs. *Daphnia magna* was more susceptible to Cefotaxime antibiotic.

3. 2 Chronic Exposures of *Daphnia magna*

The chronic toxicity data showed that Cefixime and Cefotaxime antibiotics have reproductive effects. Results for Cefixime are shown in Figure 1 as survival of test organism's and cumulative number of live offspring per surviving female during the 21 days test period. The survival % (Figure 1A) was observed to be more than 85% at the highest concentration i.e, at 56.7 mg/l. No significant difference in the first day of broods was observed (Figure 1B). The average number of neonates and number of young ones per brood were not significantly decreasing with the increase in concentration of Cefixime antibiotic in the chronic assay (Figure 1C & 1D).

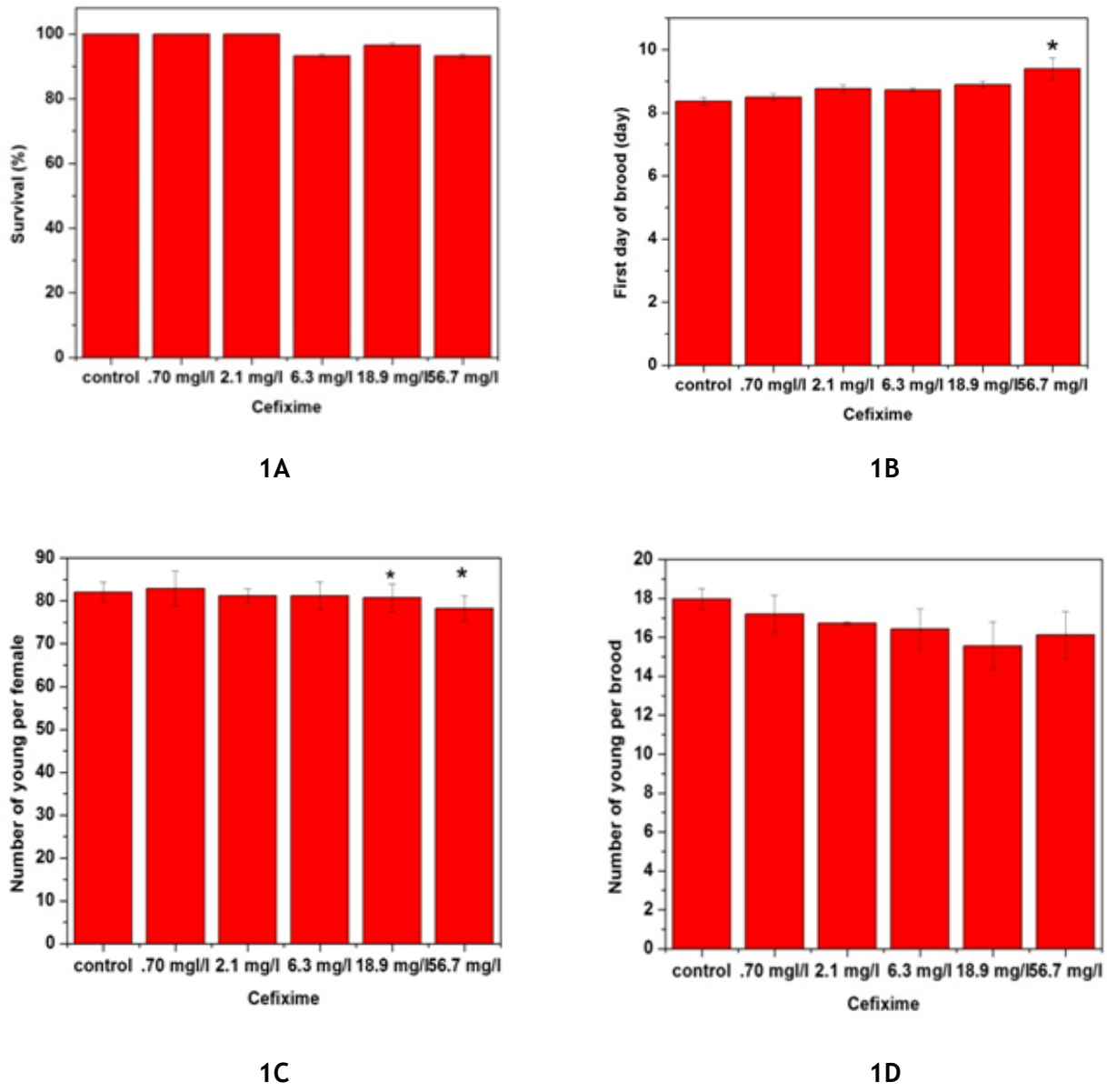


Figure 1. Results of 21 d chronic exposure to Cefixime in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different Cefixime concentrations and the control.

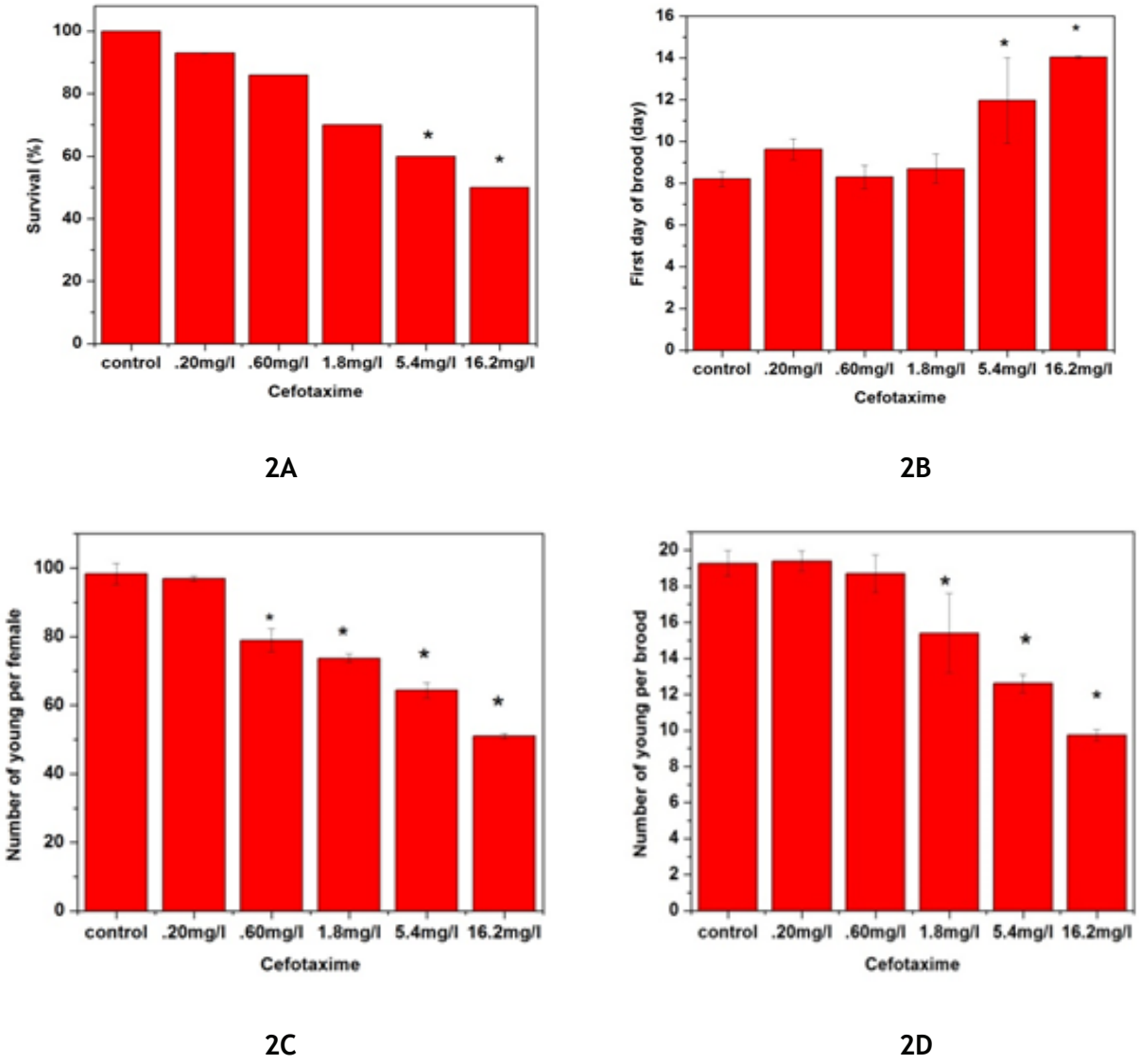


Figure 2. Results of 21 d chronic exposure to Cefotaxime in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different Cefotaxime concentrations and the control.

4. Discussion

Beta-lactams are the most common antibiotics used to treat gram - positive and gram - negative bacterial infections in humans and animals. They have a wide range of bactericidal action to kill bacteria by inhibiting the synthesis of bacterial cell walls[23]. Beta-lactams having very much chemically highly reactive and intense beta-lactam ring[21]. The antibacterial core structure of cephalosporin antibiotics was first isolated in 1945 from the fungus *Cephalosporium acremonium*[14]. The beta-lactam ring of Cephalosporin molecules is attached to a 7-aminocephalosporanic acid (7- ACA), and two main substituents located at C3- and C7-position[14], and which are responsible for the antibacterial activity. Meanwhile, the substituents at the C3- and C4-positions mainly rule pharmacokinetics[38]. They inhibit cell wall biosynthesis in bacteria by connecting to their penicillin binding

proteins inside the microbial membrane, and reaction between peptidoglycan and cephalosporins results in an irreversible inhibition of PBP, resulting in cell lysis and death[14]. Penicillins and cephalosporins accounted for nearly 60% of total consumption in 2010 increasing by 41% from 2000. These are still the most common first-line antibiotics and the main treatment for common infections around the world[40].

The *Daphnia magna* is a popular species as model in environmental toxicology[18]. They have a key position as consumers in aquatic ecosystems and are also an important source of food for small fish[30]. Daphnids have become cornerstones of systems biology research for alternative testing and methodologies, prompting their use for prescreening tests before vertebrate testing, for both practical and ethical reasons[37]. Various environmental organizations, including the US Environmental Protection Agency, the American Society for Testing and Materials, the International Organization for Standardization and the Organization for Economic Cooperation and Development have adopted standardized testing procedures using crustaceans[35]. The extensive literature on the use of *Daphnia*, as bio-indicators is due to the fact that in acute toxicity, they are considered as high sensitivity analytical tools to screening toxicity of common environmental chemicals and monitoring of effluents and contaminated waters[37]. Furthermore, the ease of culturing the crustaceans *Daphnia*, its well-studied biology, and its attributes of short development time and large brood size make this one of the oldest organism models for toxicological studies. In "traditional" ecotoxicity test, growth, reproduction, immobility and mortality of Daphnia are all well studied and are used as quantifiable parameters of its health; dose-response data can aid in predicting the consequences of exposure at other dose levels and life stages, in other species, or in susceptible individuals[36]. Various contaminants, including the "emerging pollutants", such as Endocrine Disrupting Chemicals (EDCs), Pharmaceutical and Personal Care Products (PPCPs), heavy metals and organophosphates are widely present in environment and can perturb the ecological pyramid, as well as humans[35]. Thus, monitoring some of these could help to protect the natural environment and human health. Daphnids have also been used extensively to analyse the effects and modes of action for a variety of pharmacological agents singly or in mixture. Since pharmaceuticals are designed to target specific pathways in humans or animals, when introduced into the environment they may affect the same or comparable pathways in mammals and invertebrates having the drug target homologies[37].

Found that[15] *Daphnia* had orthologs to 61% of human drug targets conserved and predicted homology (49%) with humans between the serotonin transporters. Heckmann et al.,[17] suggested thatthe potential mechanism of toxicity in *Daphnia*, after chronic exposure of ibuprofen (a non-steroidal anti-inflammatory drug) was similar to the mode of action in mammals and like in these this drug inhibited eicosanoid biosynthesis. Although mammals and crustaceans have different routes of exposure, target organs and toxic mechanisms, *Daphnia* could be a valid test model species prior to mammalian testing. Moreover, this model organism could aid in predicting pharmacokinetics and pharmacodynamics in human.

The acute test enabled the concentration range to be determined for the reproduction test. The data obtained for the tested drugs showed a significant variability with the following relative toxicity ranking: for Cefixime the EC_{50} value after 24 h were 335 and for 48 h 77.9 mg/l and for Cefotaxime were 162.71 and 25.82 mg/l respectively. The results are shown in Table 1 and 2. In a study[3], they found that calculated EC_{50} value of enrofloxacin and ciprofloxacin were 111000 and 23000 µg/l for chlorella and 69000 and 71000 µg/l for *M. macrocopa* respectively. In a study[25], it was found that the chronic effect on growth was observed at highest concentration of 83.0 mg/l of cefadroxil and 80.8 mg/l of cefadrine. The benzimidazole resulted in lower EC_{50} value in *Daphnia magna* showing higher sensitivity of fresh water macro invertebrates[9]. *Daphnia magna* showed more sensitivity to Cefotaxime, when compared to the Cefixime antibiotic.

The chronic toxicity was based mainly on reproduction test and the effect concentration was one magnitude below than the acute toxic levels. After 21 days of exposure, low mortality induced by Cefixime, reduction in the number of broods and the number of total offspring's indicates that antibiotic concentrations tested induce less chronic toxicity to *Daphnia magna* compared to Cefotaxime antibiotics (Figure 1). Upto 2.1 mg/l Cefixime

concentration no effect on survival of *Daphnia* was observed. And at concentration 56.7 mg/l 95% survival was observed.

The highest Cefotaxime antibiotic concentration induced 50% of mortality and the number of young ones per brood was observed to be decreasing in the chronic assay with the increase in concentration (Figure 2). The effect of Cefixime on first day of brood was also not pronounced while comparing with the control. With the increase in Cefotaxime concentration first day of brood was significantly reducing. No effect on number of young ones per female was observed in case of Cefixime and was significantly reducing with the increase in concentration of Cefotaxime. The effect on number of young per brood was also not affected even at highest concentrations, but was constantly reducing in Cefotaxime antibiotic treatment.

The study supported by work[6] were they have shown that emidizolen based ionic liquid cause significant reduction in first brood neonates and average neonates and also studied that such reduction in reproduction output of populations could cascade through natural fresh water ecosystems. The Cefotaxime antibiotic showed adverse effect than Cefixime on reproduction after 21-days exposure. Cephalosporins, through photo transformation results in formation of by-product which becomes more toxic. In a study conducted[41] found that Cephalexin, Cefradine and Cefotaxim becomes more toxic and less photoliable and becomes more persistent then parental drugs.

Acute toxicity testing is used in risk assessments of chemicals for humans and non-target environmental organisms. Acute toxicity study is better described as LC_{50}, EC_{50}, LD_{50}, which is defined as the dose which kills 50% of animals[1]. Acute toxicity provides guidelines on the dose to be use in more prolonged studies and it also provides the basis for which other testing program can be design. Acute toxicity test is done to identify the target organ of toxicity, to provides safety measures and monitoring guild lines for workers involved in the development and testing of test substances, to provides information needed for the dose selection in prolonged toxicity studies, to generate data containing the adverse effects of a substance on human, animal health and environment[39]. The chronic toxicity testing is used to characterise specific organs affected after repeated administration, to characterize dose-response relationships, to establish a no observable effect level. This test extends over a long period of time and it involves large groups of laboratory animals[34].

5. Conclusions

This study shows that under tested circumstances Cefixime was the least toxic then Cefotaxime on *Daphnia magna*. Both the antibiotics caused mortality of parental females in chronic exposure (21 days) to antibiotics. The effects on other end points i.e, number of young per brood, time of first brood and average number of neonate per female were also observed on whom Cefotaxime was more toxic too. Further studies are needed using many end points at both acute and chronic toxic levels to evaluate the ecotoxicity of pharmaceutical drugs and their metabolites in different species, since different species may react differently to these antibiotics due to distinct uptake routes and mechanisms.

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7. Conflict of interest

Not declared

8. References

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