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Evaluation of acute toxicity, stress and safe level of chloramphenicol and ginger to an Indian air-breathing fish, *Channa punctatus* (Bloch)

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Abstract
A maiden study was conducted to evaluate the acute toxicity and ethological stress of chloramphenicol and ginger to an Indian freshwater air-breathing fish *Channa punctatus* (Bloch). 96hr-LC_{50} dose of fish was determined by Behrens-Karber and Reed-Muench method. The safety levels of these materials were calculated by various methods. The physico-chemical parameters of experimental were found with the favourable limits. The ideal median 96hr-LC_{50} value was determined 55.14 mg L^{-1} and 3251.25 mg $fish^{-1}$ (56.5g kg^{-1}) for chloramphenicol and ginger respectively in a semi-static method. The observations indicated that the fish were more sensitive to chloramphenicol than ginger. There were large variations in the safe levels estimated by different methods for the test materials. In addition to dose and dose-time dependent increase in mortality rate, stress signs in the form of behavioral changes were observed in response to the test materials. Therefore, precautions should be taken when high concentrations of chloramphenicol and ginger are used in chronic treatment of *Channa punctatus*.

Keywords: *Channa punctatus*, Chlorzamphenicol, ginger, ethological responses, acute toxicity, safe level

Introduction
Intensive culture has been introduced to support worldwide demand of fish. A large number of feed additives are available to reinforce fish growth. Many antibiotics are used in this context. The utilization of antibiotics seems essential to check or treat fish diseases and to promote fish growth and health [1]. Chloramphenicol, Oxytetracycline and Erythromycin is used by most of the fish farmers. Chloramphenicol is used to treat bacterial infections in fish and other purposes in pisciculture [2-3]. However, critical issues are raised on the application of antibiotics due to its side effects on aquatic ecosystems. So, attention has being shifted to phytobiotics as a viable alternative to antibiotics [4]. Many workers have reported the beneficial effects of ginger as dietary additives in fish [5-6].
But, report on toxicity stress of Chloramphenicol and ginger in fishes is lacking. Therefore, *Channa punctatus* (Bloch), one of the most common Indian air-breathing fish was selected to study its acute toxicity and safe level.
The maiden study will help to establish the optimum dose of antibiotics/phytobiotics to ensure the production of healthy fish and safety for man.

Materials and Methods
Live specimens of *Channa punctatus* (Bloch) (50.0-65.0g body weight, 16.0-20.0cm) were purchased from the local market of Arrah (Bhojpur), Bihar during November 2017 to January 2019. They were acclimatized for a fortnight in Departmental Laboratory of VKS University, Arrah. The fishes were disinfected by washing properly with dilute $KMnO_4$ and then transferred to many large aquaria and fed with fish food.
The physico-chemical parameters of water used during the experimental works were determined using standard methods [7].
Range finding was conducted at first to determine the dose of test solution. Ten specimens were randomly given to Chloramphenicol (0, 40, 50…. 80 mg L^{-1}) and ginger (0, 2500, 3000.... 4500 mg $fish^{-1}$) feed and another set of control. The following methods were used to calculate LC_{50} dose from its estimation to confirmation:
1. Behren-Karber method [8, 9] and 2. Reed-Muench method [10].

The safe level estimation after 96hr exposure of test materials was determined following standard methods [11 - 14].

Statistical analysis was done with Graph Pad Prism 5 software. Data were entered in a Microsoft Excel spreadsheet. All the entries were double checked for any possible key board error. Results were presented as mean with standard deviation. The data were subjected to one way ANOVA to determine level of significance.

Results and Discussion

1. **Physico-chemical characteristics of water:** The physicochemical characteristics of test water having temperature of 22.0±2.0^{0}C, pH: 7.48±0.28, dissolved oxygen: 6.4±0.8 mg L^{-1}, total alkalinity: 76.0±4.5 mg L^{-1}, hardness: 150.6±5.2 mg L^{-1} and chloride: 16.7±0.2 mg L^{-1} was recorded during experimental period. The observed parameters are within in range for favourable growth performances has been documented [15]. Physiological parameters like concentration and formulation of antibiotics and phytobiotics and its exposure also substantially have an effect on such studies [16].
2. **Ethological observations:** Fish exposed to different doses of chloramphenicol and ginger displayed uncoordinated behavior. At preliminary exposure, fish were alert, stopped swimming and remained static in position in response to sudden alternations inside the surrounding environment. After sometime they attempted to avoid the toxic water with fast swimming and jumping. Faster opercula activity was observed as surfacing and gulping of air.

Their fins became tough and stretched due to stretching of body muscles. They secreted enormous amounts of mucus from entire body constantly and soon thick layer of mucus was found deposited inside the buccal cavity and gills. Body pigmentation of fish was increased. Ultimately fish lost balance and became unconscious, engaged in rolling movement and have become exhausted and lethargic. At last, fish exhibited vertical posture for a couple of minutes with mouth up close to the surface of the water trying to gulp air and tail in a downward direction. Soon they settled at the bottom of the tank, and after some time their bellies grew to become upward and the fish died, at the same time the opercula remained wide open exposing the gills.

Behavioral alterations as a result of stress are further standard as the maximum sensitive indication of potential toxic effects. The numerous behavioral modifications like restlessness, abnormal swimming behavior, vigorous jerks of body, loss of balance, myotonia and anorexia observed in *Channa punctatus* are like the earlier observations in fishes exposed to various toxicants [17-19].

3. 96hr-LC_{50} dose determination: LC_{50} values recorded in this study were attributed to size of fishes with potentially immune system for biotransformation of test substances from the body. Moreover, the rapid distribution of test substances in the body of fishes lead to faster alterations in behaviour than the normal for the uptake of a toxicant is directly dependent on the size of fishes.

(I) Behren-Karber method [8-9]: It is a non-parametric method. Equal spacing of the interval of dose and the equal number of fish at each dose was applied for observation from 0 to 100%.

(A) 96hr-LC_{50} dose of chloramphenicol = 55.5 mg L^{-1} (Table 1).

(B) 96hr-LC_{50} dose of ginger = 3262.5 mg $fish^{-1}$ (56.739g/kg) (Table 2).

(II) Reed-Muench method [10]: In this method, experimentation with 10 fish (mean body weight: 57.5 g) per each dose is necessary for better correlation. Further, the least test dose must kill one fish and there should be only four test doses. It leads to a bias in the estimation of the LC_{50} if the log of the dose is not spaced symmetrically about log LC_{50}. The method was modified in calculating survival and mortality of percent of test animals to arrive a conclusion [20].

(A) 96hr-LC_{50} dose of chloramphenicol = 54.20 mg L^{-1} (Table 3).

The ideal LC_{50} was calculated from the mean of MLC_{50} and $MSC_{50} = \frac{54.20 + 55.34}{2} = 54.77$ mg L^{-1} (Table 2). The difference between MSC_{50} and MLC_{50} is 55.34 - 54.20 = 1.14mg L^{-1}.

The conformation of LC_{50} was done applying three cross checks: (a) Sum of doses and sum of mortalities: Its value was $\frac{40+50+60+70}{1+3+6+9} = \frac{220}{19} = 11.59$. Multiplication of 11.59 x 5 is 57.89. Therefore, LC_{50} = 57.89mg L^{-1}. The value was close to the ideal LC_{50} value. (b) The ratio of MLC_{50} and MSC_{50}: It was found $\frac{54.20}{55.34}$ = 0.98 and 1 – 0.98 = 0.02. Multiplication of 0.02 with 54.20 is 1.08. This dose was almost equal to the value of difference. (c) Average weight of a fish (g) = 57.5 g. Therefore, 54.77 mg Chloramphenicol kills a fish of 57.5 g. Suppose, x = 1000 g. Then x = $\frac{1000.0 - 54.77}{57.5}$ = 16.44 mg L^{-1} or 54.77 – 16.44 = 38.331 mg L^{-1}. LC_{50} of a Chloramphenicol = $LC_{50} = \left(\frac{EC_{50}}{3}\right)$ x g x 10^{-4} = $\frac{38.331}{3}$ x 57.5 x 10^{-4} = 0.0735 mg L^{-1}. It shows that ideal median 96hr-$LC_{50} \sim EC_{50}$.

Thus, ideal mean lethal 96hr-LC_{50} of chloramphenicol was 55.50+54.77 = 55.14 mg L^{-1}. A dose of 50.0 mg L^{-1} of chloramphenicol has been reported for *Clarias gariepinus* [19]. Earlier, 96hr-LC_{50} dose of amoxicillin of 1000 mg L^{-1} for *Oryzias latipes* has been investigated by Park and Choi [21]. Moreover, sub-lethal effects of two antibiotics on *Danio rerio* development under amoxicillin (48hr-LC_{50} 132.4 mg L^{-1}) and oxytetracycline (72hr-LC_{50} 127.6 mg L^{-1}) showed delayed hatching [22]. Thus, toxicity of antibiotics to fish has been shown to be affected by age, size and their health [23].

(B) 96hr-LC_{50} dose of ginger = 3213.0 mg $fish^{-1}$ (55.878 g kg^{-1}) (Table 4).

The ideal LC_{50} is calculated from the mean of MLC_{50} and $MSC_{50} = \frac{3213.0 + 3267.0}{2}$ = 3240.0 mg $fish^{-1}$ (Table 4). The difference between MSC_{50} and MLC_{50} is 3267.0 - 3213.0 = 54.0 mg. The conformation of LC_{50} was done applying two cross checks: (a) Sum of doses and sum of mortalities: Its value was $\frac{2500+3000+3500+4000}{1+3+6+9} = \frac{13000}{19}$ = 684.21. Multiplication of 684.21 x 5 is 3421.05 mg $fish^{-1}$. Therefore, LC_{50} = 3421.05 mg $fish^{-1}$. The value was also close to the ideal LC_{50} value. (b) The ratio of MLC_{50} and LSC_{50}: The ratio was found $\frac{3213.0}{3267.0}$ = 0.983 and 1 – 0.983 = 0.017. Multiplication of 0.017 with 3213.0 is 54.621. This dose was almost equal to the value of difference.

Thus, ideal mean lethal 96hr-LC_{50} of ginger was 3262.50+3240.0 = 3251.25 mg $fish^{-1}$ (56.5 g kg^{-1}). A maiden work calculated 24hr-LD_{50} dose of ginger 4.5g/kg in rats [24]. Therefore, 24hr-LD_{50} dose of ginger should be at least 45 g kg^{-1} in a fish following general method of safe level from rat to fish. The finding and estimation showed closeness. It seems that toxicity of ginger to fish has been shown to be stricken by age, body weight, species and more and more on tolerance limit.

The consequences showed that chloramphenicol was more toxic to the fish than ginger. The toxicity was both time and concentration dependent.

It is reported that, safe levels are added to account for uncertainties in data and evaluation processes. The safe level is also used in case of lacking of data on acute toxicity. A range of safe level of chloramphenicol was calculated from 0.5514 x 10^{-4} to 2.757 mg L^{-1} in *Channa punctatus*. Moreover, 0.05514 x 10^{-4} to 0.2757 mg L^{-1} and 0.005514 x 10^{-4} to 0.02757 mg L^{-1} of chloramphenicol allow a safe level for rat and man respectively. Similarly, a range of safe level of ginger was calculated from 0.618 x 10^{-4} to 2.827 g kg^{-1} in *Channa punctatus* (Table 5). Moreover, 0.0618 x 10^{-4} to 0.2827 g kg^{-1} and 0.00618 x 10^{-4} to 0.02827 g kg^{-1} of ginger allow a safe level for rat and man respectively. The range indicates that it is difficult to decide the acceptable concentration of either chloramphenicol or ginger in *Channa punctatus*.

The safe levels obtained in the present study showed variations among the various antibiotics and phytobiotics. However, the large variation in safe levels calculated by various methods has resulted in controversy over its acceptability [25-26]. The underscored fact that extrapolation of laboratory records to the field is not always meaningful and subsequently it is difficult to decide on an acceptable concentration based on the laboratory experiments that may be considered "safe" in the field [27].

Table 1: Behren-Karber method for 96hr-LC50 dose of Chloramphenicol for *Channa punctatus* (body weight: 57.5 g)

Group	Dose of Chloramphenicol (mg L^{-1})	Difference between two consecutive dose (A)	No. of fish exposed	Mortality				Overall mortality at 96hr	Mean mortality Between two Consecutive dose (B)	A x B
				24hr	48hr	72hr	96hr			
1	0	0	10	0	0	0	0	0	0	0
2	40	20	10	0	0	0	1	1	0.5	10.0
3	50	10	10	0	1	1	3	3	2.0	20.0
4	60	10	10	1	3	5	6	6	4.5	45.0
5	70	10	10	2	5	8	9	9	7.5	75.0
6	80	10	10	4	7	9	10	10	9.5	95.0
										245.0

$$96hrLC_{50} = LC_{100} - \frac{\Sigma AB}{N} = 80 - \frac{245}{10} = 80 - 24.5 = 55.5 \text{ mg } L^{-1}.$$

Table 2: Behren-Karber method for 96hr-LC50 dose of ginger for *Channa punctatus* (body weight: 57.5 g)

Group	Dose of ginger (mg $fish^{-1}$)	Difference between two consecutive dose (A)	No. of fish exposed	Mortality				Overall mortality at 96hr	Mean mortality between two consecutive dose (B)	A x B
				24hr	48hr	72hr	96hr			
1	0	0	10	0	0	0	0	0	0	0
2	2500	1250	10	0	0	0	1	1	0.5	625.0
3	3000	500	10	0	1	1	3	3	2.0	1000.0
4	3500	500	10	1	3	5	6	6	4.5	2250.0
5	4000	500	10	2	5	8	9	9	7.5	3750.0
6	4500	500	10	4	7	9	10	10	9.5	4750.0
										12375.0

$$96hrLC_{50} = LC_{100} - \frac{\Sigma AB}{N} = 4500 - \frac{12375}{10} = 4500 - 1237.5 = 3262.5 \text{ mg/fish } (56.739 \text{ g } kg^{-1}).$$

Table 3: Reed-Muench method for 96hr-LC_{50} dose of Chloramphenicol in *Channa punctatus* (body weight: 57.5 g)

Sl. No.	Dose (mg L^{-1})	Log dose (mg L^{-1})	Experiment		Specific Cumulative			Rate of mortality	% mortality	% survival
			No of mortality	No. of survival	Mortality	Survival	Total			
1	40	1.602	1	9	3	36	39	$\frac{3}{39}$	7.70	92.30
2	50	1.699	3	7	6	12	18	$\frac{6}{18}$	33.33	66.67
3	60	1.778	6	4	12	5	17	$\frac{12}{17}$	70.59	29.41
4	70	1.845	9	1	21	1	22	$\frac{21}{22}$	95.45	4.55

Median lethal concentration (MLC50)	Median survival concentration (MSC50)
$\frac{50.0-33.33}{70.59-33.33} = \frac{16.67}{37.26} = 0.447$	$\frac{50.0-29.41}{66.67-29.41} = \frac{20.59}{37.26} = 0.553$
1.699 – 1.778 = 0.079	1.699 – 1.778 = 0.079
0.447 x 0.079 = 0.0353	0.553 x 0.079 = 0.0437
1.699 + 0.0353 = 1.734	1.669 + 0.0437 = 1.743
Antilog of 1.734 = 54.20 mg L-1	Antilog of 1.743 = 55.34 mg L-1
MLC50 = 54.20 mg L-1	MSC50 = 55.34 mg L-1

Sl. No.	Dose (mg L^{-1})	Log dose (mg L^{-1})	Experiment		Specific Cumulative			Rate of mortality	% mortality	% survival
			No of mortality	No. of survival	Mortality	Survival	Total			
1	2500	3.398	1	9	3	36	39	$\frac{3}{39}$	7.70	92.30
2	3000	3.477	3	7	6	12	18	$\frac{6}{18}$	33.33	66.67
3	3500	3.544	6	4	12	5	17	$\frac{12}{17}$	70.59	29.41
4	4000	3.602	9	1	21	1	22	$\frac{21}{22}$	95.45	4.55

Median lethal concentration (MLC_{50})	Median survival concentration (MSC_{50})
$\frac{50.0-33.33}{70.59-33.33} = \frac{16.67}{37.26} = 0.447$	$\frac{50.0-29.41}{66.67-29.41} = \frac{20.59}{37.26} = 0.553$
3.477 – 3.544 = 0.067	3.477 – 3.544 = 0.067
0.447 x 0.067 = 0.0299	0.553 x 0.067 = 0.0371
3.477 + 0.0299 = 3.5069	3.477 + 0.0371 = 3.5141
Antilog of 3.5069 = 3213.0 mg L^{-1}	Antilog of 3.5141 = 3267.0 mg L^{-1}
MLC_{50} = 3213.0 mg $fish^{-1}$ (55.878 g kg^{-1})	MSC_{50} = 3267.0 mg $fish^{-1}$ (56.817g kg^{-1})

Table 5: Estimation of safe levels of Chloramphenicol/ginger at 96hr exposure of *Channa punctatus* (body weight: 57.5 g)

S. No.	Method	Dose of		Accumulation Factor		Safe level	
		Chloramphenicol (mg L^{-1})	Ginger (mg $fish^{-1}$)	Chloramphenicol (mg L^{-1})	Ginger (mg $fish^{-1}$)	Chloramphenicol (mg L^{-1})	Ginger (mg $fish^{-1}$)
1.	Canadian Council of Resource and Environment Minister (=CCREM) (1991) and International Joint Commission (1977)	96hr-LC_{50} = 55.14	96hr-LC_{50} = 3251.25	0.05	0.05	55.14x 0.05 = 2.757	3251.25x 0.05 = 162.562 (2.827 g kg^{-1})
2.	National Academy of Sciences/ National Academy of Engineering (=NAS/NAE) (1973)	96hr-LC_{50}= 55.14	96hr-LC_{50} = 3251.25	0.00001 to 0.01	0.00001 to 0.0pj1	55.14x 0.01 = 0.5514 x 10^{-4} to 0.5514	3251.25x 0.01 = 32.512x 10^{-4} to 32.512
3.	Sprague (1971)	96hr-LC_{50}= 55.14	96hr-LC_{50} = 3251.25	0.01	0.01	55.14x 0.01 = 0.5514	3251.25x 0.01 = 32.512 (0.618 g kg^{-1})

Conclusion

It may be concluded that acute toxicity studies are used as the first step to determine the water quality requirements of fish that cause fish mortality. However, chemical determination of toxicant in water may not provide information on the severity of contamination, especially in the case of sublethal levels. For better result, after the use of Behren-Karber method the Reed-Muench method may be used to confirm the value of LC_{50} dose. The present study showed that chloramphenicol is more toxic to *Channa punctatus* than ginger. Therefore, precautions should be taken when high concentrations of chloramphenicol and even ginger are used in chronic treatment of fish.

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