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Experimental study on toxicity of Indian major carp bile

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Abstract

Raw bile of three Indian Major Carps viz., *Catla catla*, *Labeo rohita*, and *Cirrhina mrigala* was screened for their ichthyohepatotoxic properties. The raw bile of all the three species exhibited spontaneous haemolysis of sheep blood. Fish bioassay was conducted by intraperitoneal administration of raw bile to the test fish *Labeo rohita* of 30±5 g weight. Raw bile of all the three species studied proved to be toxic to the fish with Median Survival Time of 14 hours at 0.03 cc, 14 hours at 0.05 cc, and 18 hours at 0.05 cc of the bile of Catla, Rohu and Mrigal respectively. Behavioral and other changes included external inflammation of belly region, necrosis at the site of injection, piping, lethargy, vertical orientation, general apathy and lack of responsiveness, abdominal dropsy, and visceral hemorrhage. The results indicate Catla bile to be the most toxic followed in decreasing order by Rohu and Mrigal bile. The results are discussed in light of earlier published data and the potential risks of acute renal damage for human beings are indicated.

Keywords:
Bile, Indian major carp, Toxicity, Hemolysis

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Introduction

The fresh water grass carp is commonly eaten in Asia. Swallowing its gall bladder raw is believed by some to be good for health especially for rheumatism (Chan *et al*., 1985). Some Chinese people eat raw gall bladder of chicken, snake or fresh water fish in the belief that this will improve their usual acuity (*Chen et al.,* 1984). In China, people believe that ingestion of fish gall bladder may improve their visual acuity. However, poisoning cases are of frequent occurrence when people ingest fish bile. In a long-term, the intoxication of fish gall-bladder is seriously threatening the victims' lives and health. In the past 35 years from 1964 to 1999, according to incomplete statistics 451 persons suffered poisoning for swallowing the gall-bladder of cyprinds, among which 83 resulted in death (Wu *et al*, 2001). The death rate is 18.04%. In certain parts of India also, whole fish along with some herbal medicine is swallowed by people to cure asthma as it is a common practice in Hyderabad, India.

Crude extracts of carp bile were described to be toxic to animals (Lin *et al*.,1977; Yip *et al*., 1981). Poisoning resulting from gall bladder of fresh water carps in a Chinese population has been reported by Ngan Kum (1977), and in other areas by Yip *et al*.(1981), Chan (1976), Chen (1978) and Chan *et al*. (1985). A proposed toxin in the bile of five species of Chinese fresh water carps was named Ichthyogallotoxin by Yip *et al*.(1981).

Bile toxins from carps

Takeda *et al*., (1980), isolated a toxin from left over when a poisoning incident due to ingestion of "aria" meat and "miso" soup of carp occurred in the Kyushu district of Japan. The isolate was fat soluble and was highly toxic in dogs, but much less so in mice. Chen *et al*. (1976 a) have reported five human subjects with severe acute renal failure due to ingestion of raw grass carp bile which would have been fatal if treatment had not been applied. Subsequently Chen *et al*. (1976 b) found that acute renal damage was induced in rats by oral administration of grass carp bile whereas raw hog bile was non-toxic. Yip *et al*., (1981) did the preliminary work of isolation and purification of the bile extract of grass carp (*Ctenopharyngodon idellus*) and tested its toxicity on mice. Chen *et al*. (1984) described the renal, cardiovascular and hemolytic actions in the rat of a toxic extract from the bile of the carp. Cases of acute renal failure after eating raw fish gall bladder of grass carp was also reported by Chan *et al*. (1985). Chen *et al*. (1990) studied the plasm electrolyte changes after ingestion of bile extract of the grass carp in rats. Asakawa *et al*. (1990 b) attempted to elucidate the structure of the toxin isolated from the bile of *Cyprinus carpio* which is possible responsible for carp poisoning. Yang and Wei (1990) did preliminary studies on the bile toxin from *Ctenopharyngodon idellus*, *Cyprinus carpio*, *Aristichthys nobilis* and *Hypophthalichthys molitrix*. Mori *et al*., (1992) confirmed the presence of a toxin in bile and concluded that essentially all of the bile toxicity can be explained by the toxicity of cyprinol sulfate, a major component of carp bile. They also found that some bile acids contained in raw bile were more hemolytic than cyprinol sulfate.

It is an established fact that certain toxins are present in the bile of various fishes including the carps and these pose a potential threat to human health when consumed. In certain parts of India especially West Bengal and nearby areas, the viscera is also eaten whereby the potential risk of poisoning exists. There has been reports (Anonymous, 1994) of ingesting whole fish as medication for curing asthma in Hyderabad, India. Several cases; of acute renal failure after eating raw fish gallbladder of *Ctenopharyngodon idellus* have also been reported (Chen *et al*., 1976a). In most of the reported cases, the main symptom observed was acute kidney failure, along with damage to the liver, gastrointestinal tract, and central nervous system. In severe cases, these complications led to death (Hwang et al., 2001).

The present study is therefore designed to assess the presence of these bile toxins from Indian Major Carps which are the main food fish in India, qualitatively and quantitatively within the limited scope of this humble investigation.

Materials and Methods

Fish crude bile toxin collection

Live fish specimens of Indian major carps were collected from a community pond at Naigaon, Thane. Some ice-stored specimens were also procured from Andheri fish market. The average weights of fishes were 775 g for *Catla catla*, 850 g for *Labeo rohita* and 150 g for *Cirrhinus mrigala*. These fishes were immediately brought to the laboratory, cut open just before the bioassay experiment and the gall bladders were removed and weighed. Then the bladder was punctured with a needle and the crude bile was taken in glass vials. Any bile that was remaining was stored in a deep freezer at -20° C.

Hemolysis study

From a local slaughter house, fresh sheep blood was collected and EDTA solution was used as an anticoagulant at the rate of 5 mg/ml of blood. The blood sample was then centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded. The residue was washed with saline phosphate buffer (pH 7.5) containing 0.14 M NaCl and 0.005 M Na_2HPO_4 (Ponder, 1984) and centrifuged at 5000 rpm for 3 minutes. This supernatant was discarded and the process of washing and centrifugation was repeated again. A 5% erythrocyte suspension was then prepared following Kwapinski (1965) by adding 95 ml of saline phosphate buffer to 5 ml of above residue. To 1 ml of this 5% suspension, 1 ml of test solution (crude bile) of *C. mrigala, C. catla, L. rohita* at a concentration of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml was added and incubated at 37° C for 30 minutes. The mixture was then centrifuged at 2000 rpm for 5 minutes and the supernatant was measured spectrophotomentrically at 542 nm. The tests were conducted in duplicate. Blanks were also used adding distilled water to the erythrocyte suspension.

Fish bioassay

Labeo rohita was selected as the test fish for bioassay studies because of easy availability. Fingerlings of (average weight 30±5 g) were procured from a community pond at Naigaon, Thane. These fingerlings were then stocked in a circular PVC tank of 1000 litres capacity with adequate aeration. They were fed with palletized feeds and acclimatized in laboratory for 48 hours prior to experiments.

A set of 6 fishes were conditioned in each of aquarium tank having a capacity of 27 litres. A total of 32 aquarium tanks were set up to try out crude bile toxicity in 5 different concentrations from each species viz., *L. rohita, C. mrigala and C. catla*. Replicate sets for each set were also kept. The aquarium water was aerated continuously and water exchange was 100 % every day.

5 different concentrations of crude bile were made using distilled water. The concentrations were 0.01, 0.02, 0.03, 0.04 & 0.05 cc of crude bile made up in each

case to 1 ml with distilled water. To each individual fish 1 ml of the crude toxic preparation having the above said concentrations were injected intraperitoneally. A control set was also maintained simultaneously by injecting 1 ml of distilled water without crude bile with every set of experiments.

The activities, reactions and symptoms of the injected fishes were observed from time to time. Death of fishes at 0.25, 0.5, 0.75, 1, 2, 4, 8, 14 ±2, 24, 33 ± 3, 48, 72, and 96 hours after injection was noted. Median Survival Time (LT 50) values were calculated following the rapid graphical method of Litchfield (1949).

Results and discussion

The present study established the haemolytic and toxic properties of the crude bile of the three Indian Major Carps viz., Catla, Rohu and Mrigal. The toxic nature the bile was earlier believed to be due to the excess of Vitamin A content (Scott, 1969) but recent studies (Haslewood, 1969; Chen *et al*., 1976a) suspect the principle bile salt, 5 alpha Cyprinol, to be the toxic compound. The comparative study of haemolytic activity at 0.2 cc concentration of crude bile of the fishes showed higher haemolytic property of *C. catla* bile followed by *C. mrigala* and *L. rohita* bile. According to Kung et al. (2008), carp gallbladder contains 5α-cyprinol sulfate, a compound known for its hepatotoxic and nephrotoxic effects. Although the exact mechanism of toxicity remains unclear, mild poisoning typically results in gastroenteritis, moderate cases affect the liver and kidneys, and severe poisoning can lead to multi-organ failure. Chen *et al*. (1984) opined that bile salts caused a potent haemolysis and get the haemoglobin released from haemolysed R.B.C. and this might be responsible for the long term renal functional damage.

A toxic extract of the bile of the grass carp was shown to induce 50% haemolysis of female rat blood at a dose of 0.258 mg/ml of the bile extract as opposed to 0.625 mg/ml of Sodium Cholate (*Chen et al.,* 1984). Sodium Cholate was tested in their study because the principle bile salt in carp bile viz., 5 alpha Cyprinol (Haslewood, 1968), believed to be the toxic compound (Chen *et al*., 1976a) is very similar to cholic acid in basic structure.

The crude bile from *Cirrhinus mrigala* showed pronounced effect in a dose dependent fashion on sheep blood (Table 1). The comparative haemolytic activity at 0.2 cc of crude bile revealed a strong effect of *Catla catla* bile than the other crude bile. The complete haemolytic test of *C. catla* and *C. mrigala* could not be performed due to shortage of crude bile.

Table 1. Showing haemolytic activity of crude bile on sheep blood

Crude bile quantity (ml)	*C.mrigala*	*C.catla*	*L.rohita*
0.2	0.350	0.780	0.215
0.4	0.774	-	-
0.6	0.909	-	-
0.8	1.267	-	-
1.0	1.564	-	-

The exact mechanism by which bile salts induce haemolysis is not clear, but they may cause the release of acid hydrolases from lysosomes which may lead to the general disruption of all biological membranes, including those of the red blood cells (Weissmann, 1965). Berliner and Schoenheiner (1938) considered that 3 alpha-OH and 5 beta-H structures are essential features of haemolytic activity in steroids. The haemolytic activity also decreases as the number of hydroxyl groups increase, eg., the following order of potency was observed: Lithochlic acid (1 OH), > desoxycholic acid (2 OH) > Cholic acid (3 OH) (Palmer, 1969). Therefore, Chen *et al*. (1984) surmised that the bile extract of the grass carp contained only an active chemical responsible for haemolysis with only one or two OH group (s) which therefore differed from the suspected compound Cyprinol, such has 3 – OH groups.

The toxicological and physiological effects of ichthyogallotoxin have been extensively studied in grass carp bile. Studies have shown that ingestion of raw grass carp bile leads to reduced renal blood flow and glomerular filtration rate (Chen *et al.*, 1983), delayed gastric emptying, and increased motility of the small intestine and colon (Chen, 1984). Administration of a high dose of bile acid extract (6 mL/kg, 15%) resulted in 100% mortality in rats within 2–8 hours, accompanied by hypotension, elevated plasma potassium, hydrogen ion concentration, blood urea nitrogen, and hematocrit (Chen et al., 1990). Intravenous injection of a toxic extract caused increased renal excretion of water and electrolytes, severe hypotension, decreased cardiac output, and hemolysis (Chen et al., 1984). Hwang et al. (2000) suggested that hemolysis induced by grass carp bile may be attributed to 5α-cyprinol sulfate.

The results of the fish bioassay involving *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* bile are shown in Tables 2-4. The bile of all the three carps studied were found to be toxic to the test fish. The crude bile injected fishes showed symptoms of imbalance, piping, lying on its side of the body and floating on the water surface just before and after death. They also showed a peculiar symptom of remaining vertically in water, appearing to float with only a feeble support offered by the tail which was touching the bottom or was very close to the bottom. They remained inactive except for feeble pectoral fin and opercular movements. They also had a tendency to position themselves close to the walls at the corner or close to the air stones.

The body part of test fishes was the injection was made showed signs of muscle disintegration. The body cavity also got swollen with dark patches all along the ventral side.

The fish bioassay of *L. rohita* crude bile showed greater mortality after 14 ± hours after injection (Table. 2).

Table 2. Showing results of fish bioassay involving crude bile of *Labeo rohita*

Time intervals	No. of dead fishes at different doses				
(Hours)	0.01cc	0.02cc	0.03cc	0.04cc	0.05cc
0.25					
0.50					
0.75					
1.00					
2.00					
4.00					
8.00					II
14±2					I
24.00			I	I	I
33±3			I	II	II
48.00		I	II	III	
72.00			I		
96.00		I	I		

Table 3. Showing results of fish bioassay involving crude bile of *Catla catla*

Time intervals	No. of dead fishes at different doses				
(Hours)	0.01cc	0.02cc	0.03cc	0.04cc	0.05cc
0.25					
0.50					
0.75					
1.00					
2.00					
4.00				I	
8.00			II	I	II
14±2			I	II	III
24.00			II	II	
33±3					I
48.00					
72.00			I		
96.00					

The doses of 0.01 and 0.02 showed the least lethal effect. But the higher doses of 0.03, 0.04 and 0.05 cc wiped out the test fishes within 72 hours after the injection. The Median survival Time (LT 50) was found out to be 14 hours at 0.05 cc dose (Fig. 2). The dose of 0.01 and 0.02 cc did not bring about death upto 96 hours (Table 3). The injected fishes showed peak mortality between 33± 3 hours and 72 hours. The Median Survival Time (LT50) was found out to be 14 hours at 0.03 cc dose (Fig. 2). At 0.01 cc dose, all the injected fishes survived 96 hours duration of the experiment (Table. 4) and the effect of crude bile did not express itself except for some ulceration at the body part where the injection was made. At 0.03 cc dose half the no. of the injected fishes died but they started dying after 24 hours along with the fishes injected with 0.04 and 0.05 cc dose. All the fishes died within 33±3 hours in case of 0.05 cc dose and 48 hours period in case of 0.04 cc dose. The Median Survival Time (LT50) was found out to be 18 hours at 0.05 cc dose (Fig. 4)

In the fish bioassay, the Catla bile took 14 hours (LT 50) at a concentration of 0.03 cc to kill the test fish (Fig. 1) whereas Rohu registered LT 50 values at 14 hours (0.05cc) while Mrigal bile showed LT 50 values 18 hours at 0.05 cc (Fig. 3). Thus, it is observed that Catla bile was the most toxic to the fishes, followed in decreasing order by the bile of Rohu and Mrigal. Mortality effected after 24 hours was not taken into consideration following convention. Hwang et al. (2000) purified and identified 5α-cyprinol sulfate and its desalted form, 5α-cyprinol, from grass carp bile using infrared spectroscopy, NMR (^{1}H and ^{13}C), and

Table 4. Showing results of fish bioassay involving crude bile of *Cirrhinus mrigala*

Time intervals (Hours)	No. of dead fishes at different doses				
	0.01cc	0.02cc	0.03cc	0.04cc	0.05cc
0.25					
0.50					
0.75					
1.00					
2.00					
4.00					
8.00					I
14±2					I
24.00			I	II	II
33±3			II	III	II
48.00			II	I	
72.00			I		
96.00					

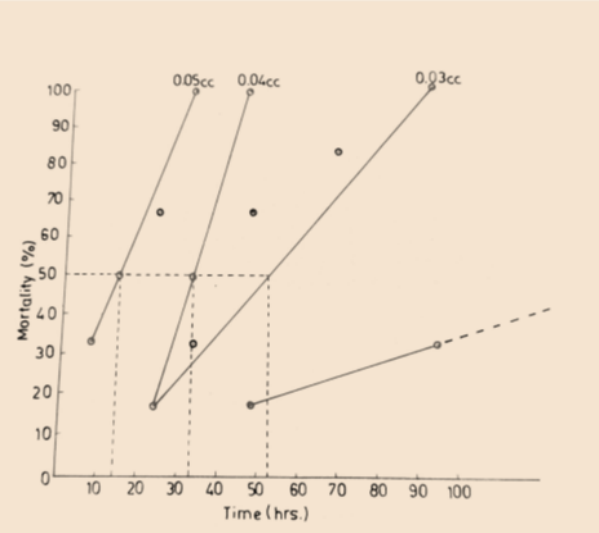

Fig. 1: Toxicity of raw bile of Rohu to test fish

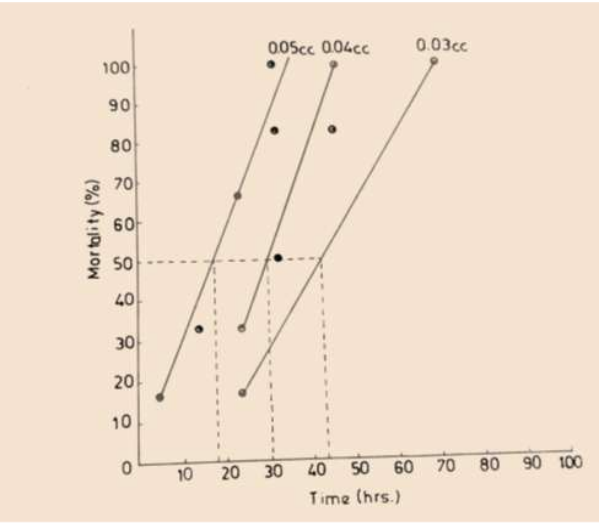

Fig. 2: Toxicity of raw bile of Catla to test fish

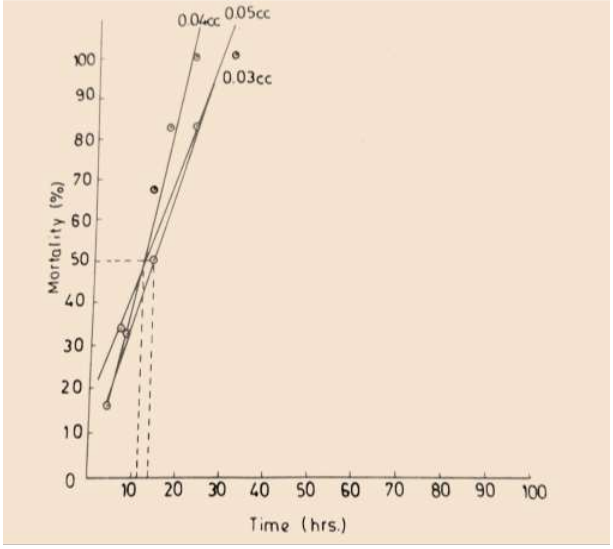

Fig. 3: Toxicity of raw bile of mrigal to test fish

mass spectrometry. Toxicity studies in rats revealed that grass carp bile powder, its butanol extract, and 5α-cyprinol sulfate significantly impaired kidney and liver functions. While 5α-cyprinol also affected renal function, its toxicity was comparatively lower.

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