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Analysis of the toxic factor present in tor Sp. of Chalakudi River, Western Ghats, India

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Abstract
An unidentified rare mahseer species of the Chalakudy river is a red variety locally known as 'choppan choora' is not eaten by the people as its consumption leads to giddiness, nausea and vomiting which last for at least 24 hours. Bioassay-guided fractionation was employed to identify the active compounds from fish sample. Purification of the crude extract was done by Gel filteration chromatography. Spectrophotometric analysis of *Tor* sp. showed highest peaks as compared to the *Tor khudree sykes, 1839.* Water sample analysis of toxic cyanobacteria showed negative result by PCR. Preliminary studies on mouse Bioassay was carried out at Central Institute of Fishery Technology, Kochi. Fraction showed mild symptoms in Aqueous extract. Intraperitoneal mice study was conducted at Kerala Veterinary and Animal Sciences University, Thrissur. Mice exhibited lethality within 24hr. Control was maintained throughout the study from non toxic *Tor sp.*

Keywords: Toxic Factor, Red Mahseer, Chalakudy River, *Tor sp.*

1. Introduction
Indian Mahseer is the big scaled carps and also a major species in the catch of the up-stream areas. It has an important fishery in the Himalayan water bodies-India, tributaries and impoundments are the cradle of the Indian Mahseer. The unique ecological dynamism prevailing in this system might be supporting the Mahseer significantly for their distribution and existence. Western Ghats is the important habitat for Mahseer. Langer *et al.* (2001) [6] described this group as the 'King of Indian aquatic systems'. Population of these species shows a sharp declining trend in many rivers. High food value and exemplary sport value make this group extraordinarily important to any fisheries scientist. Its meat is tasty and the texture is appealing. On the contrary, there are reports (mainly from Sri Lanka) on the presence of toxins in certain species of mahseer. Similar reports, though many decades old and very vague in nature, are also available from some parts of India [5]. Until now such reports are not found in Kerala. Recently the Kadar group of tribes reported that a specific variety of mahseer caught from the up-streams of Chalakudy River contains a toxin which makes them inedible [5]. The whole body of Red Mahseer is reddish in colour with the high level of pigmentation on the scales and also in the opercular region. The eyes have a reddish ring surrounding the cornea. The scales are also slightly red in colour. The embedded portion of the scales is black. This is the one and only fish in the Western Ghats of Chalakudy River which has been reported to have a toxic factor in it. The main aim of the study is to investigate the problem in detail as part of the study and find out the probable reason for the toxicity.

2. Materials and Methods
2.1 Sample collection
Fish samples were collected from two places of Chalakudi, Kerala *viz.,* Mukkampuzha and Pokalappara. Study period is from 01.02.2012 to 31.01.2013. Pokalappara colony has been protected by trenches and electrical fencing to prevent the entry of wild animals while Mukkampuzha is an unprotected colony. The fishes from the sampling centres were collected by hook and line with the help of tribes. Samples were collected in a self-sealing polythene bag and were transported to the lab in insulated box filled with ice. The samples on arrival in the lab were packed into small quantities, labeled properly and then stored in a deep freezer (-20 ^{0}C). Care was taken to minimize repeated thawing and freezing.

Water samples were collected in 1L polythene bags for the analysis of toxic cyanobacteria.

2.2 PCR Analysis for cyanobacteria

PCR was performed using the modified protocol of Nubel *et al*. (1997) [8]. It was used in the present study for cyanobacteria detection. The total DNA in the water samples (Sample -1 is 25.2ng/µl and Sample -2 is 126.4ng/µl) was extracted using Invitrogen genomic DNA extraction kit. The DNA concentrations of the samples were measured using Nanodrop spectrophotometer at 260nm wavelength. Cyanobacterial specific primers, forward primer Cya 359F (5'-GGGGAATYTT CCGCAATGGG-3') and the reverse primer Cya 781Ra (5'GACTACTGGGGTATCTAATCCCATT-3'), or the reverse primer Cya781Rb (5' GACTACAGGGGTATCTAATCCCTTT-3') or an equimolar mixture of reverse primers Cya781Ra + Cya 781Rb19. Reaction mixtures contained 75 picomoles of each primer, 25 nmol of each deoxynucleoside triphosphate, 10µL of 10xPCR buffer (100 mM Tris –HCl [pH9.0], 15 mM $MgCl_2$, 500 mM KCl, 1% [v/v] Triton X -100, 0.5µl of SuperTaq DNA polymerase and template DNA. A known positive sample and negative sample were included. Amplifications were carried out in 100µl volumes in a Perkin-Elmer/ Cetus DNA. Thermal Cycler with a single cycle of 1 min at 94 ^{0}C, 1 min at 58^{0}C, and 1 min at 72 ^{0}C, followed by 24 incubation cycles each consisting of 1 min at 94 ^{0}C, 1 min at 58 ^{0}C, and 1min at 72^{0}C followed by 15 min extension of 72 ^{0}C. Aliquots of the resulting 448bp sequences were electrophoresed in 1.5% agarose gels containing 10 µgmL-1 ethidium bromides and documented through a UV illuminator.

Total plate count for cyanobacteria was measured by the method of AOAC (2005) [2]. To 100µl sample 0.3µl of dye SYTO (Green fluorescent nucleic acid stain) was added and incubated for 20 min. Direct Count was taken in Haemocytometer under epifluroscence microscope.

2.3 Methods of analysis of fish sample

2.3.1 Preparation of the crude extracts

Five samples, each weighing 5.0 g, were respectively extracted with Aqueous, Acetone, Hexane, Methanol, and chloroform for 2 h, and the process was repeated for three times. The ratio of sample to solvent was 1:10 (m/v). Each extract was subsequently centrifuged at 14000 rpm for 10 minutes at 4 °C and filtered (0.25µm filter). Four filtrates (other than Aqueous) were concentrated under reduced pressure in a vacuum rotary evaporator until a constant yield was obtained and then kept at -20 ^{0}C. These four extracts were thawed and fed to the mice.

2.4 Purification by Gel Filtration

Aqueous extract sample was further purified by gel filtration on Sephadex G-100 column got eluted at 0.1M phosphate buffer concentration according to the method of Andrews (1970) and Porath and Flodin (1959) [1, 9]. Swollen gel was prepared by 2.5 g Sephadex G-100 (Sigma Chemicals Co., St. Louis, Missouri, USA) was taken in a beaker and allowed swelling in 100 ml distilled water for 24 hr. The solution was stirred and allowed to settle. The supernatant was decanted; washing was done two more times to remove the fine particles. The column was washed first with 2 column volumes of distilled water (started with 0.2ml/min and slowly increased to 0.5ml/min). The column was equilibrated with 2 column volumes of buffer (0.1M phosphate buffer at pH 7). The internal diameter of the column should be 1cm. Mount the column vertically on a laboratory stand. A carpenter's level was used to determine that the column is vertical. Column was filled with distilled water and a cotton plug was introduced at the bottom after opening the stopper. An appropriate volume of the gel suspension was poured, in order to fill completely the 40 cm column bed height. The gel suspension was poured onto a glass rod whose end touches the inner column wall. This will result in a smooth flow of the gel suspension without unnecessary turbulence and introduction of air. Since the volume of the slurry usually exceeds the desired column bed volume, it is convenient to use a gel reservoir or column extension to hold the excess slurry. The column was connected to a reservoir containing phosphate buffer of pH 7. This buffer was passed through a column at a flow rate of 10 ml /hr for about 8 hr. Then it was stopped and applied 1 ml of the sample (Aqueous extraction). The buffer reservoir was connected and continued the elution. 1 ml fraction was collected for 60 minutes (10 fractions were collected). The purified fraction was stored at -20ºC until further use. Elution profiles of the fraction were monitored by measuring absorbance at 280nm. The fractions with maximum absorbance were pooled and allow to rotary vacuum flash evaporator to get a dry powder. The crude extracts were dissolved in 10 ml saline solution (0.9% NaCl) to obtain 0.125 gml−1 stocking solutions which were used for the preparations of the desired concentrations for each extract and the control groups were set up under the same conditions as the test groups (Wang, *et al.,* 2009) [12]. UV absorption is perhaps the simplest method of measuring the concentration of proteins in solution. A typical protein absorption spectrum has an absorption peak at 280nm, due to the aromatic amino acids, such as tryptophan and tyrosine. UV absorption is the method favoured for continuous monitoring of the protein concentration in the eluate from chromatography columns (Dennison, 2003) [4]. Ultraviolet light absorption at 280 nm was used to monitor the appearance of protein in the column eluate (Boyd, 1974) [3]. Quantifying protein by directly measuring absorbance is fast and convenient, since no additional reagents or incubations are required. No protein standard need be prepared and the procedure does not consume the protein. The relationship of absorbance to protein concentration is linear.

2.5 Mouse bioassay

2.5.1 Oral study

Mouse Bioassay was performed by the method of Rhodes *et al*. (2002) [10]. Animal feeding was carried out under Central institute of fishery technology (CIFT), Kochi. Male Swiss albino mice weighing 25-30g body weight were used for the study. The animals were housed in polypropylene cages (with stainless steel grill top) under hygienic and standard environmental conditions (28±2^{0}C, Humidity- 60-70%, 12:12 light/dark cycle). The animals were allowed a standard diet (M/S Sai feeds, Bangalore India) and water ad libitum. The experimental animals were divided into 2 groups of 10 cages comprising 2 mice each. Totally 20 mice were used for 10 extraction. The crude extracts (Dry powder) were dissolved in 10 ml saline solution (0.9% NaCl) to obtain 0.125 gml−1 stocking solutions. One group fed with toxic fraction other group fed with non-toxic fraction for 2 days. The sample was administered to mice orally at a uniform volume of 0.3 ml mice-1 the animals were observed for 24 hr after dosing. Next

day volume was increased to 0.6ml mice -1.

2.5.2 Intraperitoneal study

Study was carried out in the laboratory of KVASU (Kerala Veterinary and Animal Sciences University) Thrissur. 0.025ml of the fraction was injected intraperitoneally into five 18-21g male BALB/c mice. The animals were observed for signs of toxicity for 24hr after dosing. Next day, the dose was increased to 0.25ml. The tests were carried out only to get a rough idea about the toxic factor under study**.**

3. Results

3.1 Screening of the samples for toxic cyanobacteria

Water samples collected from two different areas of Chalakudy river viz., Pokalapara, Mukkampuzha, were used in the present study. Collected sample were screened for detecting the toxic cyanobacteria by PCR. Water sample Analysis of cyanobacteria in Mukkampuzha and Pokalapara areas Total Plate Count was 3.6x105 cfu/ml, 1.8x105 cfu/ml and Direct count was 2.24x108cells/ml, 2.14x107cells/ml. PCR showed Negative against toxic cyanobacteria of both study areas. Species identified in Mukkampuzha (study area) were 1. *Rhizobium radiobactor*, 2. *Bacillus cereus*, 3. *Acinetobacter calcoaceticus*, 4.*Chrysobacterium balustinum*, 5.*Klebsiella pneumonia*, 6.*Proteus vulgaris*, 7.*Photorhabdus luminescens*, 8.*Enterobacter aerogenes*, 9.*Myroides odoratus*, 10.*Bacillus sphaericus* and Pokkalapara (study area) were 1.*Pseudomonas balearica*, 2.*Acinetobacter lwoffii*, 3.*Pedobacter heparinus*, 4.*Sphingomonas sanguinis*, 5.*Proteus vulgaris*, 6.*Photorhabdus luminescens* 7.*Myroides odoratus*, 8.*Klebsiella pneumonia*, 9.*Grimontia hollisae*, 10.*Proteus mirabilis*, 11.*Bacillus sphaercius*. The results showed that there were no toxic cyanobacteria in the water. Microorganisms were counted by total plate count and direct count method. These results showed that microorganisms present in the water were at a normal range. No harm to water or fishes (study area) was attributed due to the microorganisms, because of the normal count noticed. Figure 1.

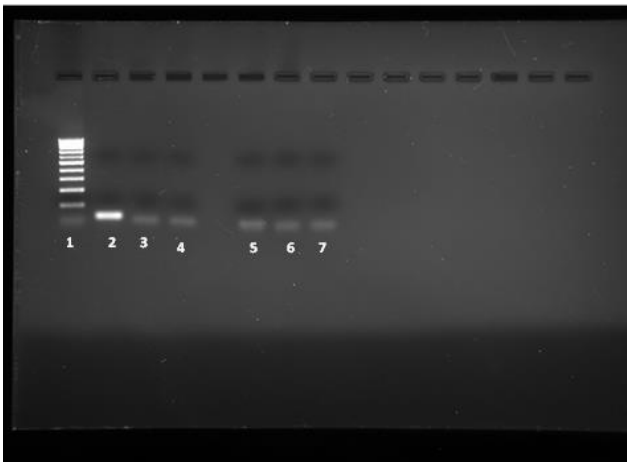


Fig 1: shows that Agarose gel electrophoresis of samples screened for toxic cyanobacteria by PCR 1.5% Agarose gel. Lane 1- Marker; Lane 2- Sample 1 reverse primer a; Lane 3- Sample 2 reverse primer a, Lane 4- Negative reverse primer a; Lane 5- Sample 1 reverse primer b; Lane 6- Sample 2 reverse primer b; Lane 7- Negative reverse primer b (Sample1 – Mukkampuzha area and Sample 2- Pokkalapara area).

3.2 Elution profile of sample

Purification of the crude protein was done by Gel filteration chromatography on a Sephadex G-100 column and after purification the elution profile was monitored by spectrophotometry at 280 nm. Peak was eluted by 0.1 M Phosphate buffer (pH 7.0). The highest peaks of fraction were collected and concentrated through Rotary vacuum flash evaporator and tested for toxicity in mice. Figure 2.

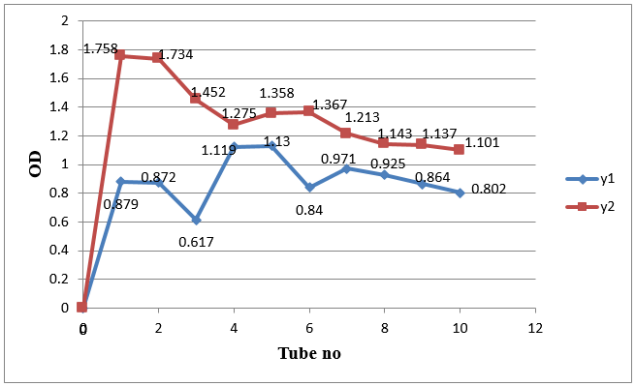
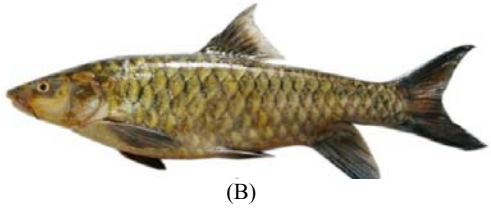


Fig 2: Aqueous fraction of y1 and y2 samples (y1 – Control (*Tor khudree* sample non toxic), y2 – Test (Red mahseer sample-Toxic).

The result in Figure 2 showed that Spectrophotometric study of the material in aqueous fraction, 3 peaks in both red mahseer and *Tor khudree* samples. The toxic Red mahseer sample shows the highest peak as compared to the non toxic *Tor khudree* sample. Figure 3.

(A)



(B)

Fig 3

The result in Figure 3 showed that samples of control and test species *ie* a] *Tor Khudree b*]Red Mahseer Figure 4.

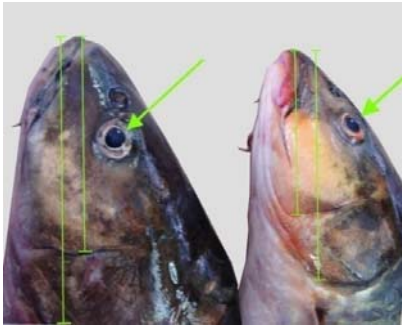


Fig 4

The result in Figure 4 showed that morphological difference between control and test species *ie* 1] *Tor Khudree* (Left to Right) *2*] Red Mahseer.

3.3 Mouse bioassay

The fractions were administered orally into five groups of Male Swiss albino mice with 25 to 30g body weight. Mice were observed and monitored for 2 days with 2 different doses (0.3ml and 0.6ml) for five extractions of both the sample. There was a mild toxic symptoms (lethargy and hunching) observed in mice through oral route administration of Aqueous Extract at 0.6ml dose. The mice became lethargic, with a hunched appearance, soon after dosing and by 15 minutes they became immobile. Recovery began after 1 hour and by 3 hours, the appearance and behaviour of the mice became normal.

Mice were injected intraperitoneally into 5 mice of 18-21g male BALB/c mice for five different fractions. Mice were observed and monitored for 2 days with 2 different doses (0.025ml and 0.25ml). Fractions exhibited lethality to mice at 0.25ml dose in 30 minutes for aqueous fraction and 45minutes for methanol fraction and 42 minutes for chloroform fraction and 60 minutes for acetone fraction and 63 minutes for hexane fraction. This observation proved that aqueous extraction separate the toxic factor could more effectively. Restlessness, gasping for breath, exploring around the cage, standing on the hind legs *etc.* were the various symptoms shown by the mice before death.

4. Discussion

Toxicological evaluation of natural sample by chromatographic fractioning improves the performance for the detection of toxic compounds in sample. In the current study also, the extraction steps were carried out meticulously so that the toxic factor could be effectively taken out. There are ever so many toxins associated with the fish consumption and its side effects. Yessotoxin is an example, which shows no diarrheic effects in humans besides its severe toxicity. The current toxic factor under investigation also does not show any diarrheic effect in humans. This was proved in the mouse bioassay, as the oral administration of the toxic factor to the mice did not evoke any such responses. Pectenotoxins also do not cause diarrhoea in humans. Its oral administration to mice does not cause any toxic effect, in spite of the toxicity induced by intraperitoneal administration (Miles *et al.,* 2004; Suzuki *et al.,* 2006) [7, 11]. A similar result was also obtained in the current toxin. Oral feeding did not cause any mortality but the i.p. injection caused instant mortality. It is true that this kind of observations present a very confused biochemical intervention of the toxin. Only through detailed studies extending to decades, one can find out the mysteries behind such issues. While the toxicity of the extracts of five solvents were compared (Aqueous, Acetone, Hexane, Chloroform and Methanol), the aqueous extract showed the maximum potency in mice. Cyanobacterial blooms potentially affect water quality as well as the health of human and animal life. Decomposition of large blooms can lower the concentration of dissolved oxygen in the water, resulting in hypoxia (low oxygen) or anoxia (no oxygen). Wild animal poisonings can occur after ingestion of cyanobacterial biomass and intake of toxins during drinking and feeding. In the case of current research the toxic cyanobacteria were not found in the study area. The result of GFC (Gel Filtration Chromatography) demonstrate that clean-up has removed a significant amount of matrix interference from the total extract, thereby increasing the sensitivity of detection generally by 2 to 5 times relative to that before the GFC step. But GFC eliminated these problems of biological matrix thus resulting in a much cleaner and better resolved chromatogram. Spectrophotometric analysis showed that, material in aqueous fraction, 3 peaks in both Red mahseer (*Tor* sp.) and *Tor khudree* sample. The red mahseer sample showed that the highest peak as compared to the *Tor khudree*-non toxic sample. Test solution was injected orally into 25 to 30g Male Swiss albino strain mice. Mice were observed and monitored 2 days with 2 different doses (0.3ml and 0.6ml) for five extractions of both sample. Mild toxicity symptoms were observed through oral route. The mice become lethargic, with a hunched appearance, soon after dosing and by 15 minutes they were immobile. Recovery began after 1 hour and by 3 hour the appearance and behaviour of the mice was entirely normal. Test solution was injected intraparitoneally into 18-21g Male BALB/c mice. Mice were observed and monitored 2 days with 2 different doses (0.025ml and 0.25ml) for five extracts. Lethality was observed within an hour. A control was maintained throughout the study using a normal fish without any undesirable compounds. The control presented no ill effects in all the studies.

5. Conclusion

The toxin in the Red Mahseer, is interesting from the biochemical and academic point of view. It is assumed that the current toxin is a proteinaceous compound. Serious vomiting has been the most significant symptom noticed. Even though a few cases are hospitalized, no mortality has been reported so far. Need further studies for exactly characterizing the toxic factor and follow up its course of biochemical pathways.

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