



ISSN (E): 2277- 7695

ISSN (P): 2349-8242

NAAS Rating: 5.03

TPI 2019; 8(6): 807-810

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www.thepharmajournal.com

Received: 11-04-2019

Accepted: 15-05-2019

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Investigation of acute toxicity and behavioural changes on *Oncorhynchus mykiss*, Rainbow trout fry in response to ethanolic extract of *Myrica esculenta*

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Abstract

Myrica esculenta commonly known as 'Kaphal' is regarded as an important Indian medicinal plant which possesses various pharmacological activities like antimicrobial, antiallergic, and anxiolytic. It is found in foothill tracks of the eastern Himalayas including India, Meghalaya, Nepal, China and Pakistan. Local tribes use this plant for the treatment of cough, asthma, fever, inflammation. There is no report of acute toxicity of *Myrica esculenta* in fish. The present study aimed to determine the 96 hrs LC₅₀ (Lethal Concentration) of ethanol-based crude leaf extract of *Myrica esculenta* on the fry of *Oncorhynchus mykiss*. The fishes were distributed in seven treatment groups with each group containing 20 fishes of average weight 1.1 ± 0.05 grams and length 5 ± 0.1 cm. The different treatment concentration T1 (150 mg l⁻¹), T2 (175 mg l⁻¹), T3 (200 mg l⁻¹), T4 (225 mg l⁻¹), T5 (250 mg l⁻¹), T6 (275 mg l⁻¹), T7 (300 mg l⁻¹) were given to fish through immersion as described in OECD guidelines. The 96 hrs LC₅₀ of the ethanolic extract against *Oncorhynchus mykiss* was found to be 199.52 mg l⁻¹. In the present study, the behavioural response of the fish was also recorded. The fish exhibited the jumping movement and asphyxiation in concentrations above than 175mg l⁻¹.

Keywords: *Myrica esculenta*, antimicrobial, antiallergic. LC50

1. Introduction

The *Oncorhynchus* species belong to family salmonid, commonly known as Rainbow trout, have a native distributed in Pacific coast of North America and Russia, but has been cultivated and introduced as food and game fish around the world. Rainbow trout is one of the oldest fish in culture. Some rainbow trout are also known as steelhead, meaning that they begin life in fresh water, travel to the ocean for further development and then return back to freshwater to spawn [1]. 7 distinct subspecies of Rainbow trout have been identified, these include: Coastal rainbow trout, several species of redband trout, golden trout [1]. *O. mykiss* has the highest growth rate in 15-18% salinity, slower growth in lower salinities and the lowest growth rate in freshwater [2]. *O. mykiss* were able to acclimatise to seawater during winter, although not extremely cold [3]. In India, rainbow trout is cultured in Jammu and Kashmir, Himachal Pradesh, Uttarakhand and Sikkim.

The species *Myrica esculenta* belong to the family Myricaceae, commonly known as 'Kaphal, Boxberry', consists of about 97 species of the small tree and aromatic shrubs. The only species found between 900-1200 m above the sea level in India, different species are found all over the world like- *M.persylvanica* Mirb (North America), *M. rubra* (China, Japan), *M.australiasica* F. Muell (Australia) [4]. *Myrica esculenta* is a well-known medicinal plant having diverse pharmacological properties, such as antiasthmatic, anticancer, anti-inflammatory, antioxidant and anti ulcer activities. *Myrica esculenta* is used in both Ayurvedic and Yunani system of medicines for curing diseases [5]. The flowering season starts from February and continues till the second week of April while the fruiting was observed during the first week of May. Recent studies have shown that they have strong antibacterial, antidiarrheal, anthelmintic activity of *Myrica esculenta* [6]. The purpose of the current study is to investigate the 96 hrs LC₅₀ of the ethanolic leaf extract of *Myrica esculenta* via bath treatment and examine the behavioural changes in the early stage of rainbow trout fry, *Oncorhynchus mykiss* for generating baseline information. The information can be utilized by researchers for harnessing the pharmacological activities of *Myrica esculenta* in a fish model.

2. Materials and Methods

2.1 Experimental site

The present experiment was carried out at Wet lab facility of the Mycology and parasitology laboratory of ICAR-Directorate of Coldwater Fisheries Research, Bhimtal.

2.2 Experimental set up

The fry of Rainbow trout (*Oncorhynchus mykiss*) of size 4-4.5 cm (Average weight 1 gm) were collected from trout hatchery, ICAR-DCFR, Bhimtal. The Fishes were held in well-aerated aquaria (58 cm x 31cm x 31cm) (Fig. 3) filled with 20 L tap water and allowed to acclimatized for 15 days before starting the experiment. During the acclimatization period, fish were fed with protein-rich diet.

2.3 Collection of plant and preparation of ethanolic plant extract

The *Myrica esculenta* (Fig. 2) plant was collected from the local area of Bhimtal, Uttarakhand. Briefly, the dried leaf of the plant was grinded in a fine powder by the grinder and were extracted by using ethanol as solvent.⁽⁷⁾ described that the ratio of plant powder and solvent (ethanol) was 1:10 and kept for 48 hours in a rotary shaker. The mixture was filtered through Whatman filter (No. 1) paper and centrifuged at 2460g for 10 minutes. The filtrate was evaporated in the rotary evaporated under vacuum at 40°C. At last, the extract was dried in the freeze drier for 24 h. The extract was kept at 20°C till further use⁽⁸⁾.

2.4 Acute toxicity study

2.4.1 Range-finding test

The test was conducted in aquaria through the water to determine the concentration of the ethanolic crude extract in the short-term test and for finding out LC₅₀. The fry of *Oncorhynchus mykiss* were exposed to the range of different

concentration of crude extract 10, 100, 200, 400, 600, 800, 1000 mg l⁻¹. Percentage of fry Mortality was observed and recorded for 96 hours among these different concentrations. The fry were randomly distributed into 7 groups with each group containing 15 fishes.

2.4.2 Median lethal concentration (LC₅₀) experiment

On bases of the result of range-finding test, fish were exposed to an ethanolic extract of *M. esculenta* at 150, 175, 200, 225,250 275, 300 mg l⁻¹ for 96 hours. The behavioural changes like-breathing behaviour, swimming pattern, morbidity, mortality etc were reported at different time intervals up to 96 hours. Dead fish were removed from the experimental tanks and dead fishes percentage were recorded. No water exchange and feeding were done during the whole experiment. The fry were randomly distributed into 7 groups with each group containing 20 fishes.

2.5 Water quality monitoring

At 24 hours interval, the water quality monitoring was done during the experiment. A digital thermometer was used to measure the temperature of water and dissolved oxygen, pH, ammonia, alkalinity and free carbon dioxide were analyzed by water quality analysis kits (Provided by Aqua check Himedia Laboratories Pvt. Ltd.).

2.6 Statistical analysis

The LC₅₀ values were used to determine the acute toxicity effect using the Probit method by SPSS version 16.0⁽⁹⁾.

3. Results

96 hrs LC₅₀ determination

The number of fish deaths recorded at different concentrations of the plant extract are shown in Table 1.

Table 1: Recorded number of fish death at various concentrations of the ethanolic extract of *Myrica esculenta*

Group	Total	Dose (mg/l)	Log dose	Dead	Dead %	Corrected percentage	percentage Probits
1	20	150	2.18	2	10	10	3.72
2	20	175	2.24	7	35	35	4.61
3	20	200	2.3	9	45	45	4.87
4	20	225	2.35	11	55	55	5.13
5	20	250	2.39	12	60	60	5.25
6	20	275	2.43	15	75	75	5.67
7	20	300	2.47	18	90	90	6.28

The LC₅₀ value of the ethanolic extract of *M. esculenta* extract in rainbow trout fry was found to be 199.52 mg/L (Fig.1).

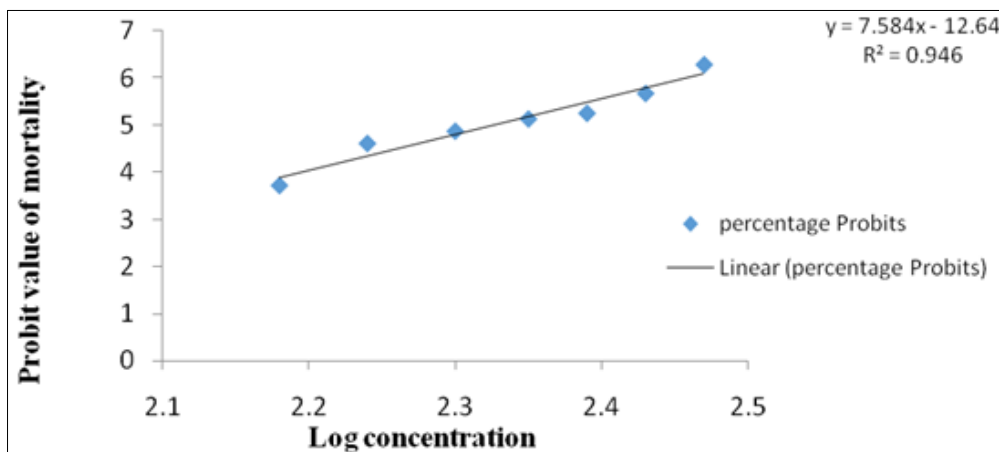


Fig 1: Plot of log concentration of plant extract vs probit values



Fig 2: Leaves of *Myrica esculenta*



Fig 3: Experimental Setup

Water quality analysis

During the toxicity tests, the average value of DO was 7.16

mg/L, CO₂: 3mg/L, pH: 6.97, temperature: 20.27 °C, alkalinity: 17.78 mg/L, ammonia: 0.03 mg/L.

Table 2: Values of DO, CO₂, pH, temperature, alkanity and ammonia of the water; at the beginning during and at the end of the toxicity tests with different aqueous *Myrica* extract concentrations (150, 175, 200, 225, 250, 275, 300 mg/L).

Concentration (mg/L)	DO (mg/l)	CO ₂ (mg/l)	pH	Temperature (°c)	Alkanity (mg/L)	Ammonia (mg/L)
150	6.70-7.36	2.0-4.0	6.65-7.13	19.8-20.6	70-100	0.01-0.03
175	6.70-7.70	2.0-4.0	6.70-7.22	19.7-20.5	70-100	0.02-0.04
200	6.64-7.72	2.0-4.0	6.60-7.31	20.1-20.5	80-120	0.01-0.05
225	6.64-7.74	2.0-4.0	6.62-7.36	20.2-20.6	80-120	0.03-0.04
250	6.66-7.70	2.0-4.0	6.62-7.38	20-20.6	80-150	0.01-0.05
275	6.64-7.72	2.0-4.0	6.60-7.38	20.1-20.5	70-175	0.01-0.02
300	6.64-7.72	2.0-4.0	6.58-7.44	20-20.6	80-200	0.01-0.05

Behavioural patterns of fish

The fishes exposed to different concentration of the ethanolic extract of *Myrica esculenta* showed behaviour changes which were observed only at 24 hrs after the addition of fresh extract. The fishes became alert, stopped swimming and remained static in position in response to sudden changes in the surrounding environment. Frequent surface to bottom movements and faster opercula activity were observed as surfacing and gulping of air increased with increase in the concentration of plant extract, it was also observed that the fishes remained in vertical position for a few minutes with terminal mouth up near the surface of water trying to gulp air, and the tail was in the downward position. Soon the fishes settled down to the bottom of the tank and were found lying at the aquarium bed before they died.

Discussion

LC₅₀ is an aqueous chemical activity which cause 50% mortality in the exposed population of fishes. Calculation of LC₅₀ are based on two assumptions, first assumption is exposure time associated with specified LC₅₀ is sufficient to allow complete equilibration between the fish and water, the Second assumption is specified LC₅₀ is minimum dose that kills the fish during the exposure interval.

The 96 hrs LC₅₀ test was conducted to measure the susceptibility and survival potential of fishes to particular toxic substances, Higher LC₅₀ value indicates lower toxicity because greater concentration is required to cause 50% mortality in fishes.

In this study, the toxicity effect of the ethanolic extract of *Myrica esculenta* was studied in terms of susceptibility, survival potential and behavioural pattern of rainbow trout fry. The susceptibility of fry to aqueous *Myrica* extract at various concentration and different exposure period showed

the increase mortality in fish fry as the concentration of extract increases. With high concentration of 275 and 300 mg/L, the high fish mortality was recorded.

These finding agreed with the other reports which showed 96 hrs LC₅₀ of garlic extract through bath treatment is 253 mg/L for *Cyprinus carpio* [10]. Further reports showed the use of *Nicotiana tobaccum* leaf extract on *Clarius garipenus* which presented the LC₅₀ of 626 mg l⁻¹ [11]. There are some other reports related with seed extract of the plants used in fishes; for an example, seed extract of *Moringa oleifera* on the *Cyprinus carpio* which presented the LC₅₀ of 12.40 mg/L [12]. The seed extract of pawpaw (*Carica papaya*) on Nile tilapia (*Oreochromis niloticus*) presented LC₅₀ of 4.2 mg/L [13], which is low as compared to other plant extracts. The 96 hrs LC₅₀ of plant extract depends on the fish species, the solvent used for extraction, route of administration and experimental conditions.

The fishes in treatment groups with high concentration of plant extract showed the signs of asphyxiation, jumping and high opercular movement. The reports suggest that excess of plant extract can get deposited in the gills, reducing gaseous and ionic exchange [14]. The addition of plant extract has led to alterations in behavior of fish after 24hrs, which is consistent with the findings of several reports that revealed the clinical and behavioural pattern after 24 hrs of experiment. [10, 15].

There is a necessity to treat the disease by exploring the various plant extracts that are harmless and long-lasting. The plant extracts are used for the treatment of fish diseases as they are non-toxic and eco-friendly.

Acknowledgements

The authors are thankful to the Director, ICAR-Directorate of Coldwater Fisheries Research Bhimtal for providing the

necessary facilities for the study.

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