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EFFECT OF *MIKANIA CORDATA* ON NON-SPECIFIC IMMUNE RESPONSE AND SURVIVAL OF *LABEO ROHITA* AGAINST *APHANOMYCES INVADANS*

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ABSTRACT

The study was conducted to evaluate the efficacy of *Mikania cordata* leaf powder on non-specific immune response and disease resistance of *Labeo rohita* fingerlings against the *Aphanomyces invadans* infection. *M. cordata* extract was incorporated in the diets (at 0.0%, 1%, 2% and 3%) of *L. rohita* fingerlings (19±0.95g). Blood and serum sampling was carried out on 0th day, 14th day, 28th day and 42th day of feeding trials to determine NBT levels, myeloperoxidase activity, phagocytic activity and serum lysozyme activity, as compared to the control group. Fish were challenged with *Aphanomyces invadans* after 42 days and mortalities were recorded over 18 days post infection. The results revealed that fishes fed with *Mikania cordata* extract showed significant ($p<0.05$) increase in NBT levels, myeloperoxidase activity, phagocytic activity and serum lysozyme activity when compared to the control group. Dietary *M. cordata* extract of 2% showed significantly ($p<0.05$) higher protection relative percentage survival (RPS 71.06± 5.773%) from *A. invadans* infection than control. These results indicate that *M. cordata* leaf powder stimulates the non-specific immunity and makes *L. rohita* more resistant to fungal infection (*A. invadans*).

Key words: *Mikania cordata, Aphanomyces invadans, Labeo rohita,* immune response

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is one of the most destructive diseases of both fresh and brackish water farmed and wild fish which caused major fish losses in many countries for three decades (Baldock *et al*., 2005). The disease is caused by an oomycete fungus, *Aphanomyces invadans* (Mohan and Shankar, 1995; Lilley *et al*., 1998; Thompson *et al*., 1999; Johnson *et al*., 2004). More than 100 fish species are reported to be affected by it (Lilley *et al*., 1998) and until recently, EUS is an important issue in the carp culture ponds (Ahmed and Hoque, 1999; Lilley *et al*., 2002; Khan and Lilley, 2002; Islam *et al*., 2003; Nandeesha and Karim, 2006) particularly during the winter months. Fingerlings of Indian major carps (IMC) suffering from heavy mortalities during natural outbreaks (Roberts *et al*., 1989; Chinabut and Roberts, 1999; Khan and Lilley, 2002) and artificial infection experiments (Mohan, 2002) have been reported. Interestingly, during EUS outbreaks in several Southern (Vishwanath *et al*., 1997a; Vishwanath *et al*., 1997b; Jayaraman, 1991) and Northeastern states of India (Kumar *et al*., 1991; Barman *et al*., 2012), IMC in many water bodies had been observed to be unaffected. High temperature in south India has been suggested as a possible explanation for the lack of disease outbreak (Roberts *et al*., 1994). However, the temperature theory alone may not support some of the observations made in the Northeastern states of India, where temperature was ideal for EUS outbreak (Pradhan *et al*., 2008). The possibility of age or size influencing the susceptibility of IMC to EUS was suggested by (Lilley *et al*., 1998; Chinabut and Roberts, 1999).

Phytotherapy is the oldest form of healthcare known to man-kind. Bioactive substance present in herbs is well-known to have an antimicrobial and immunomodulatory properties. Globally, plant extracts are employed for their antibacterial, antifungal and antiviral activities. It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine (Odugbemi, 2006) particularly in the developing countries. Herbs are an interesting alternative because they are inexpensive, renewable, locally available, user friendly and can be easily prepared (Harikrishnan and Balasundaram, 2005). Recently, there has been an increasing interest in the modulation of the non-specific immune system of fish, as a prophylactic measure against disease. Many of the medicinal plants such as *Ocimum sanctum* (Logambal *et al*., 2000), *Acalypha indica*, *Phyllanthus niruri*, *Azadirachta indica*, *Piper betle*, *Mentha piperita* (Dinakaran, 2001), *Allium sativum* (Sahu *et al*., 2007), *Astragalus membranaceus*, *Lonicera japonica* (Ardo *et al*., 2008) and *Withania somnifera* (Sharma *et al*., 2010) have been shown to trigger innate immune system and enhance disease resistance against pathogenic organisms. An extensive work on the use of immunostimulatory herbs in fish was conducted by various researchers and they suggested that

the herbal extracts can be used in fish culture as an alternate to the chemotherapeutic agents (Raman and Rahman, 2002; Raman, 2007; Kumar *et al*., 2012; Kumar *et al*., 2013).

Mikania cordata (Bumr.f.) B.L. Robinson is locally known as Refugee lata, Assam lata, German lata and Tara lata belongs to the family *Asteraceae* (Ahmed *et al*., 2008; Nayeem *et al*., 2011). It is a fast growing, creeping woody perennial climbing hempvine (Mercado, 1994). The stem and its branches and the mature leave easily form roots when these come in contact with the soil (Holm *et al*., 1977). *Mikania* (*Asteraceae*) species are found throughout tropical regions of Africa, Asia (Bangladesh and India) (Patar and Yahaya, 2012), Brazil and South America (Argentina, Paraguay and Uruguay) (Chowdhury *et al*., 2011). The family *Asteraceae* consists of several important medicinal plants with wide range of biological activities and interesting phyto-chemical constituents. Various plants of *Asteraceae* used in the management of gastrointestinal complication in traditional medicine (Hérida *et al*., 2005). Leaves of *Mikania cordata* exhibited significant antifungal activity in fish (Kumar *et al*., 2015) and antiulcer activity in rats (Paul *et al*., 2000) so it can be possible that it helps in faster healing of fungal affected tissues.

Herbals in recent years have been used as immunostimulant and therapeutic agents and because of their eco-friendly role they are given more importance in aquaculture. *A. invadans* the fungal pathogen and causative agent of EUS is almost impossible to control in fish populations and there is no protective vaccine or effective drug/chemical treatment against it. The present experiment was designed to evaluate the efficacy of *M. cordata* leaf powder on the non-specific immune response and disease resistance of *L. rohita* fingerlings against the *A. invadans* infection.

MATERIALS AND METHODS

Fish collection and maintenance: *Labeo rohita* (Hamilton, 1822) fingerlings of average length 15±0.79 cm and weighing 19±0.95 g, were collected from Don Bosco Fish Farm, Bishramganj, India. The fishes were acclimatized in FRP (fiber reinforced plastic) circular tanks of capacity approximately 500 liters, at ambient temperature (26-28 ^{0}C) with continuous aeration. They were fed twice daily with a diet (rice bran and mustard oil cake in the ratio of 1:1) at the rate of 4% body weight at 6.00 a.m. and 6.00 p.m. respectively. The optimum physico-chemical parameter of water i.e. dissolved oxygen (6.88±0.56 mg l^{-1}) and pH (7.14±0.77) were maintained throughout the experimental period.

Mikania cordata: The plant of *M. cordata* was collected from the local farmers of South Tripura District, India and the identification was done by the Botany Department, Tripura University, Agartala, India. The Plant was submitted in the form of herbarium as a voucher specimen to the botany department. The leaves was collected from the plants and washed thoroughly with tap water to rid them of dirt. After washing, the leaves were dried under shade to make them suitable for grinding. The dried plant leaves were grounded in a mechanical grinder and sieved. The powder obtained was stored in an air tight container for further use (Sharma *et al*., 2010).

Preparation of experimental diets: The experimental diet was prepared with the locally available ingredients containing 1%, 2%, 3% of *M. cordata* leaf powder (Table 1). Initially all ingredients were mixed thoroughly by adding water, pelleted by a hand pelletizer (Xie *et al*., 2008) and then dried at 40 ^{0}C for 12 hours. The dried pellets were stored in an air sealed container and stored in a cool dry place for further use.

Table 1. Composition of control and experimental diets

Ingredients/100 g of feed	Control	1 % (T1)	2 % (T2)	3 % (T3)
Wheat flour (g)	60	59	58	57
Fish meal (g)	35	35	35	35
Vitamin-mineral mix (g)	3	3	3	3
Cod liver oil (ml)	2	2	2	2
M. cordata leaf powder (g)	0	1	2	3

Experimental design and feeding diet: The experiment was performed in 500 L FRP (fibre reinforced plastic) tanks. The fishes were divided into four groups (Control, T1, T2 and T3), in triplicates with 60 fish per replicate. The control group diet was devoid of leaf powder. The experimental groups T1, T2 and T3 were fed with feed containing 1%, 2% and 3% of *M. cordata* leaf powder. Fishes were provided with adequate aeration and fed at the rate of 3% of body weight twice a day in the 6:00 a.m. and 6:00 p.m. The experiment was conducted for 42 days and the sampling for various immunological parameters was carried out on days 0, 14, 28 and 42. For each sampling 8 fishes were selected randomly from each tank and analyzed for various parameters.

Collection of blood from the fish and separation of serum: Blood was collected using sterilized 2 ml hypodermal syringes and 24 gauge needles washed with 2.7% EDTA (Qualigens, India) as an anticoagulant. Blood was drawn from the caudal peduncle region. Before drawing blood, fishes were anaesthetized with clove oil (Merck, Germany). For serum separation the blood was similarly collected without anticoagulant in serological tubes and stored in a refrigerator overnight. The clot was then spun down at 3000 x g for 10 min. The serum collected was stored in sterile serum tubes at -20

^{0}C until used for assays. All the procedures were carried out in the sterilized condition. After drawing blood fishes were given 1% $KMnO_4$ dip treatment and released in to the tank.

Non-specific immune parameters

Nitroblue tetrazolium assay: Nitroblue tetrazolium (NBT) assay was determined by the method of Secombes (1990) as modified by Stasiack and Baumann (1996). 50 µl of blood was placed into the wells of flat bottom microtitre plates and incubated at 37 ^{0}C for 1 h to facilitate adhesion of cells. Then, the supernatant was removed and the loaded wells were washed three times in PBS and 50 µl of 0.2% NBT was added and was incubated for 1 h. The cells were fixed with 100% methanol for 2-3 minutes and again washed thrice with 70% methanol. The plates were then air dried and 60 µl 2N potassium hydroxide (KOH) and 70 µl dimethyl sulphoxide (DMSO) were added into each well to dissolve the formazan blue precipitate formed. The OD of the coloured solution was read at 620 nm (BioTek, Power wave 340, ELISA reader, India).

Myeloperoxidase activity: The myeloperoxidase activity present in serum was measured according to Quade and Roth (1997) with slight modification by Sahoo *et al*. (2005). About 10 µl of serum was diluted with 90 µl of Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in flat bottom 96-well plates. Then 25 µl of 20 mM '3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) (Himedia, India) and 25 µl of 5 mM H_2O_2 (Qualigens, India) (both substrates of MPO and prepared on same day) were added. The colour change reaction was stopped after 2 min by adding 50 µl 4 M sulphuric acid (H_2SO_4). Plate was centrifuged at 400 x g for 10 min, and 150 µl of the supernatants, present in each well, were transferred to new 96 well plates. The OD was read at 450 nm in an ELISA reader (BioTek, Power wave 340, ELISA reader, India).

Serum lysozyme activity: Serum lysozyme activity was measured using colorimetric method by Anderson and Siwicki (1995). In a cuvette, 3 ml of *Micrococcus luteus* (ATCC 7468, India) suspension in phosphate buffer (A_{450} = 0.5-0.7) was taken, to which 50 µl of diluted serum sample was added. The content of cuvette was mixed well for 15 s and measured using a spectrophotometer at 450 nm. The reading of lysis of the bacteria was immediately recorded at interval of 15, 30 and 270 s. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 per minute and lysozyme activity was expressed as U/min.

Phagocytic activity (PA): Phagocytic activity was detected using *Staphylococcus aureus* (Bangalore Geni, India) as described by Anderson and Siwicki (1995). A sample (0.1 ml) of blood was placed in a microtiter plate well and 0.1 ml of *S. aureus* 1 x 10^7cfu ml^{-1} (A_{450} = 0.5-0.6) cells suspended in phosphate buffered saline (pH 7.2) was added and mixed well. The plate was incubated for 20 min at room temperature. 5 µl of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air-dried, then fixed with ethanol (95%) for 5 min and airdried. The air-dried smear was stained with 7% Giemsa for 10 min. Two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the numbers of phagocytizing cells were counted. Phagocytic activity equals the number of phagocytizing cells divided by the total number of phagocytes counted.

PA = Number of phagocytizing cells x 100/Number of total cells

Preparation of fungal spores: *L. rohita* affected with *A. invadans* obtained from local fish farmer ponds. The affected muscle (approx. 2 mm^3) were dissected and placed on a Petri dish containing the isolation medium (Glucose peptone agar medium) (Lilley *et al*., 1998). Inoculated media are incubated at approximately 25°C and examined under an inverted microscope within 12 hours. Emerging hyphae tips were repeatedly transferred to fresh plates of GP agar until cultures are free from bacterial contamination. After four days, the agar from the resulting fungal mat was washed out by sequential transfer through five petri dishes containing autoclaved pond water (APW) and mats were kept in a petri dish containing 25 ml of (APW) at 20°C. After about 12 hr, the motile secondary zoospores were collected and number of zoospores in the suspension was counted (6 x10^4 spores per ml) using haemocytometer (Pradhan *et al*., 2008).

Challenge with A. invadans spore: After 42 days of feeding with *M. cordata* supplemented diet, 10 fishes from each replicate were selected randomly. The experimental fish were injected intramuscularly (into the left flank of fish just below the middle of dorsal fin region) with 0.1 ml of spore suspension (6 x10^4 spores per ml) of *A. invadans* as described by Chinabut *et al*. (1995). Control fish groups were treated with 0.1 ml autoclaved pond water at the same time. After injection, of experimental and control groups were kept separately in 500 l capacity fiberglass tubs containing 400 l water. The fishes were observed regularly for any overt signs of disease including behavioural abnormalities and mortality. Sampling of the survivors was carried out on the 14th day of *A. invadans* infection. The causative agent was confirmed by re-isolating *A. invadans* from the moribund fish.

Relative percentage survivals (RPS) were calculated accordingly as follows:

$$\text{Relative percentage survivals (RPS)} = \frac{\text{Number of surviving fishes after challenge}}{\text{Number of fishes injected with } A.\ invadans} \times 100$$

(Misra *et al*., 2006)

Statistical analysis: The data was statistically analysed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan's multiple range test (DMRT) was used to determine the significant differences between the means. Comparisons were made at 5% probability level.

RESULTS

Immunological parameters: NBT level in all the groups of fishes fed with diet containing *M. cordata* leaf powder at various levels showed significant ($p < 0.05$) difference in nonspecific immune responses on days 14, 28 and 42. The NBT activity (OD at 620 nm) of the experimental groups were found to be significantly ($p < 0.05$) different in the treatment groups when compared with control and observed highest in T2 group on all sampling days (Fig 1). The myeloperoxidase activity (OD at 450 nm) of the experimental groups increased significantly ($p < 0.05$) in the treatment groups and showed an increasing trend from days 14 to 42 of sampling (Fig 2). The highest myeloperoxidase activity was found in treatment group T2 followed by T1 and T3.

The level of lysozyme activity in all groups (T1, T2 and T3) of fishes fed with diet containing *M. cordata* leaf powder increased from day 14 to 42 and then decreased noticeably (Fig 3). Lysozyme levels in group T2 were significantly ($p < 0.05$) higher on days 14, 28 and 42. Phagocytic activity in T1, T2 and T3 groups of fishes fed with diet containing *M. cordata* leaf powder showed increasing trend from days 14 to 42 then decreased noticeably. The group T2 was observed to be significantly higher ($p < 0.05$) compared to other treatments on all sampling days.

Relative percentage survival: Relative percentage survival of *L. rohita* after challenge with *A. invadans* in different experimental groups is presented in Fig. After injection with *A. invadans*, the first mortality was recorded after 8 days. The treatment groups fed with *M. cordata* leaf powder supplemented diet showed significantly ($p < 0.05$) high disease resistance against *A. invadans* infection when compared with control group. The highest percentage survival was recorded in T2 (71.06%) followed by T1 (60.95%) and T3 (49.84%) groups (Fig 5).

DISCUSSION

Herbs, which also act as immunostimulant, stimulate the innate defense mechanisms and provide protection in fishes (Pandey *et al*., 2012; Barman *et al*., 2013; Kumar *et al*., 2014). The current research is directed towards an alternative approach- the use of herbs to boost or stimulate non-specific immune response as well as protection against *A. invadans*. The increase in non-specific immune parameters and resistance against *A. invadans*, causative agent of EUS in *L. rohita* fingerlings after administration of *M. cordata* leaf powder through feed have been reported for the first time in *L. rohita*. As analternative to chemotherapy, application of natural products, like plant extracts, in aquaculture is new and developing venture which needs further research in fish (Citarasu *et al*., 2002; Jian and Wu, 2003; Sivaram and Babu, 2004; Kumar *et al*., 2016).

The respiratory burst (NBT) activity can be quantified by the Nitroblue Tetrazolium (NBT) assay, which measures the quantity of intracellular superoxide radicals produced by leukocytes (Sahu *et al*., 2007; Ardo *et al*., 2008). Herbal based immunostimulants can enhance the respiratory burst activity of fish phagocytes. In the present experiment the experimental groups fed with *M. cordata* the supplemented diet has higher NBT activity as compared to the control group. For instance, Rao *et al*. (2006) reported that Superoxide anion production by the blood leucocytes was enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed. Ardo *et al*. (2008) also reported that feeding Nile tilapia (*Oreochromis niloticus*) with two herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells. Myeloperoxidase (MPO) is a peculiar and specific hemeprotein released by neutrophils. It is secreted and functional during activation of neutrophils, which plays an important role in the defence of an organism. MPO is abundantly stored and expressed in primary azurophilic granules of neutrophils. It utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid (Dalmo, 1997). In the present study, *M. cordata* the supplemented dietary fed groups showed higher myeloperoxidase activity in comparison to control. Kumar *et al*. (2015) demonstrated that fishes fed with *M. cordata* extract showed significant increased in NBT levels and myeloperoxidase activity when compared to the control group. Siwicki (1987) reported that *Cyprinus carpio* injected with levamisole showed increased myeloperoxidase activity. Similar increase in MPO was reported by Kumari and Sahoo (2006), *Clarius batrachus* fed with β-1, 3 glucan and in *L. rohita* injected with curcumin (Behera *et al*., 2011). Similarly, higher myeloperoxidase activity was observed in *Oplegnathus fasciatus* fed with vitamin-E (Galaz *et al*., 2010).

Phagocytic activity is a key indicator of enhanced non-specific immune response. In the present study the increase in phagocytic activity in the treatment group signifies the role of *M. cordata*in enhancing the

nonspecific immune response. Similar increase in phagocytic activity was reported by Asmi *et al*. (2002) in *Cyprinus carpio* fed with oligodeoxynucleotides supplemented diet. Greasy groupers (*Epinephelus tauvina*) fed with herbal diet containing purified active component of *Ocimum sanctum*, *Withania somnifera*, and *Myristica fragrans* (Sivaram and Babu, 2004), chinese sucker (*Myxocyprinus asiaticus*) fed with traditional Chinese medicinal plant extracts (Zhang *et al*., 2009) have increased phagocytic activity. The higher phagocytic activities in the treatment groups might be due to activation of phagocytic cells mostly neutrophils and monocytes in the circulation and *M. cordata* might have also activated the complement factors via the alternative pathway, which acts as opsonin leading to enhancement of phagocytosis.

In the present study, serum lysozyme was significantly increased in all experimental groups. The present observation was similar to findings of Chen *et al*. (2003) who reported that plasma lysozyme activity was increased in crucian carp by feeding four Chinese herbs (*Rheum officinale, Isatis indigotica* and *Lonicera japonica*). The level of serum lysozyme was also enhanced in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed (Rao *et al*., 2006). Elevated lysozyme was also observed in Japanese eel (*Anguilla japonica*) after feeding with Korean mistletoe extract (KM-110; *Viscum album* Coloratum) (Choi *et al*., 2008). It is generally accepted that Lysozyme is a humoral component of the non-specific defense mechanism that has the ability to prevent the growth of infectious microorganism by splitting β-1, 4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls (Alexander and Ingram, 1992; Gopalakannan and Arul, 2006; Choi *et al*., 2008).

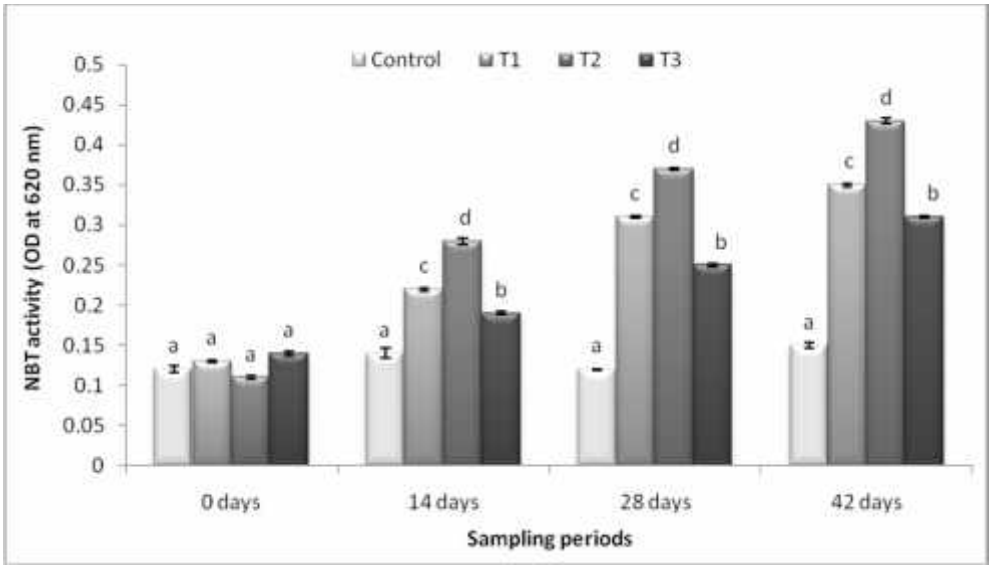

Fig. 1 NBT activity in *L. rohita* differently fed with 0% (control), 1%, 2% and 3% of *M. cordata* (values are mean ± SE). a,b,c,d different letters indicate significant difference between different treatment groups within a sampling day ($p < 0.05$) (n= 60 fish/replicate).

The challenge test with *A. invadans* showed increased relative percentage survival in groups treated with *M. cordata*. This might be due to the enhancement of the non-specific immune system of fish by herbal plant extracts. In agreement with the present findings, Kumar *et al*. (2015) reported that *M. cordata* leaf powder significantly increased non-specific immunity and decreased mortality in *C. catla* experimentally infected with *A. invadans*. Similar finding was observed by Sahu *et al*. (2007) reported that survival rate after challenging the fish with *A. hydrophila* was enhanced in *Labeo rohita* fed diets containing *Magnifera indica* kernel. Ardo *et al*. (2008) showed that feeding with two Chinese medicine herbs and challenging with *A. hydrophila*, increased the survivability in tilapia (*Oreochromis niloticus*) Pachanawan *et al*. (2008) also reported that survival rate after challenging the fish with *A. hydrophila* was increased in tilapia (*Oreochromis niloticus*) fed diets containing either dry leaf powder of *Psidium guajava* or ethanol extract of *P. guajava* leaf. In addition to this *M. cordata* leaf powder supplemented feed also provides resistance against *A. invadans* infection and reduces mortality in *L. rohita*. The response of the dose 2 g kg^{-1} in the present observations was maximum, might be the most appropriate dose which activated the receptors and the corresponding genes responsible for the secretion of immune defence factors.

The study concluded that *Mikania cordata* leaf powder increase the non-specific immunity and significantly decrease mortality when *L. rohita*

experimentally infected with *A. invadans*, a fungal pathogen. The study opens up new approaches for future study on most effective dose under pond conditions, degree and duration of the resistance offered, administrative regime for different age group of fish and time of application to ensure improved harvest in culture ponds. Moreover, further studies are needed to determine the effect of *M. cordata* in other animals or in humans, molecular mechanisms involves in the process and isolation and characterization of the active compounds/ ingredients responsible for antifungal activity of the plants *M. cordata* in fish.

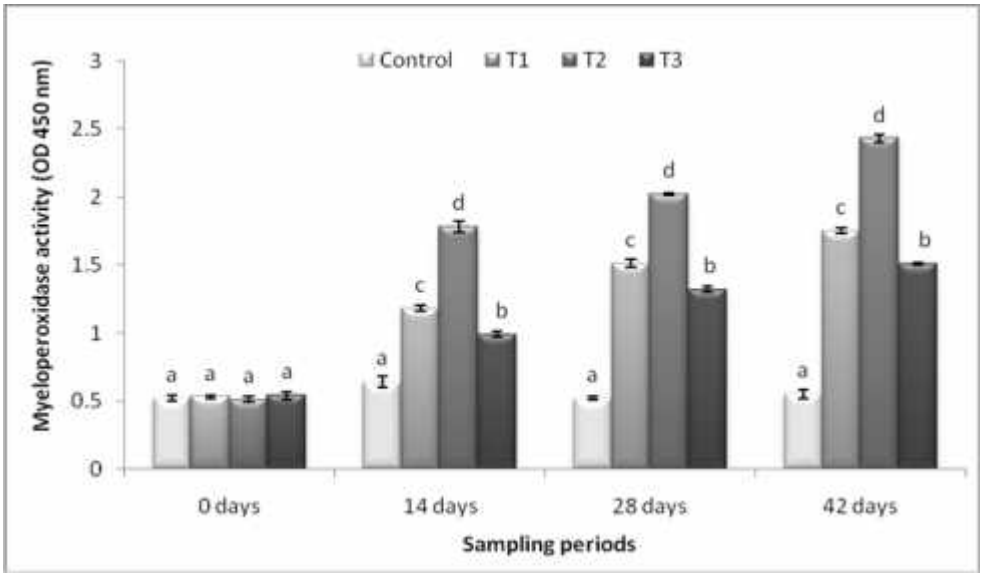

Fig. 2. Myeloperoxidase activity in *L. rohita* differently fed with 0% (control), 1%, 2% and 3% of *M. cordata* (values are mean ± SE). a,b,c,d different letters indicate significant difference between different treatment groups within a sampling day ($p < 0.05$) (n= 60 fish/replicate).

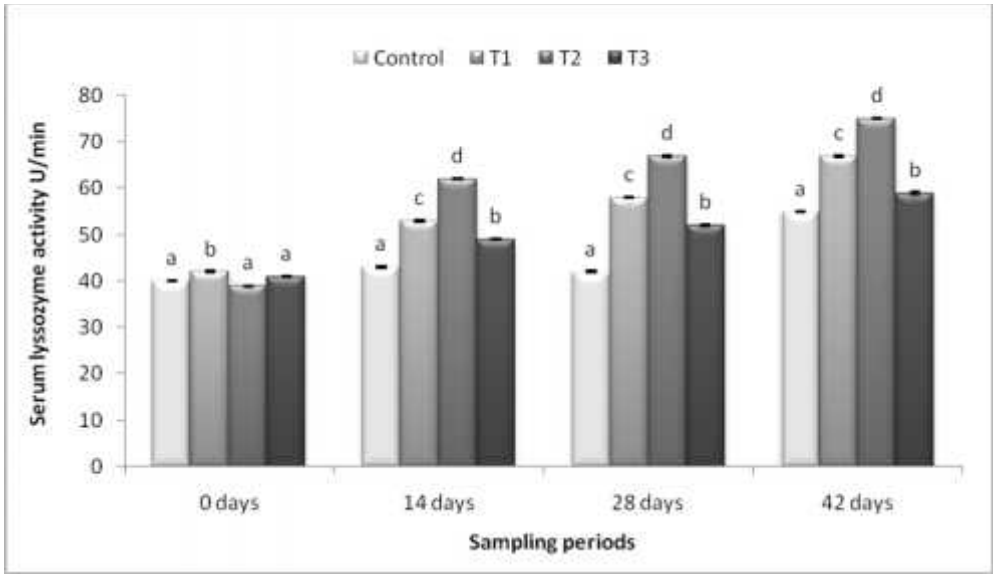

Fig. 3. Serum lysozyme activity in *L. rohita* differently fed with 0% (control), 1%, 2% and 3% of *M. cordata* (values are mean ± SE). a,b,c,d different letters indicate significant difference between different treatment groups within a sampling day ($p < 0.05$) (n= 60 fish/replicate).

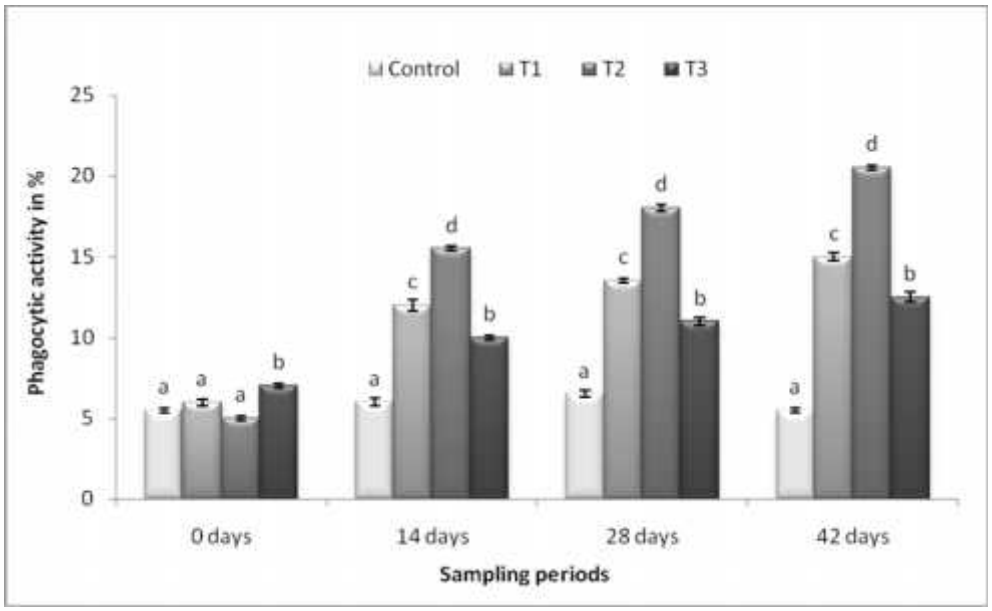

Fig. 4 Phagocytic activity in *L. rohita* differently fed with 0% (control), 1%, 2% and 3% of *M. cordata* (values are mean ± SE). a,b,c,d different letters indicate significant difference between different treatment groups within a sampling day ($p < 0.05$) (n= 60 fish/replicate).

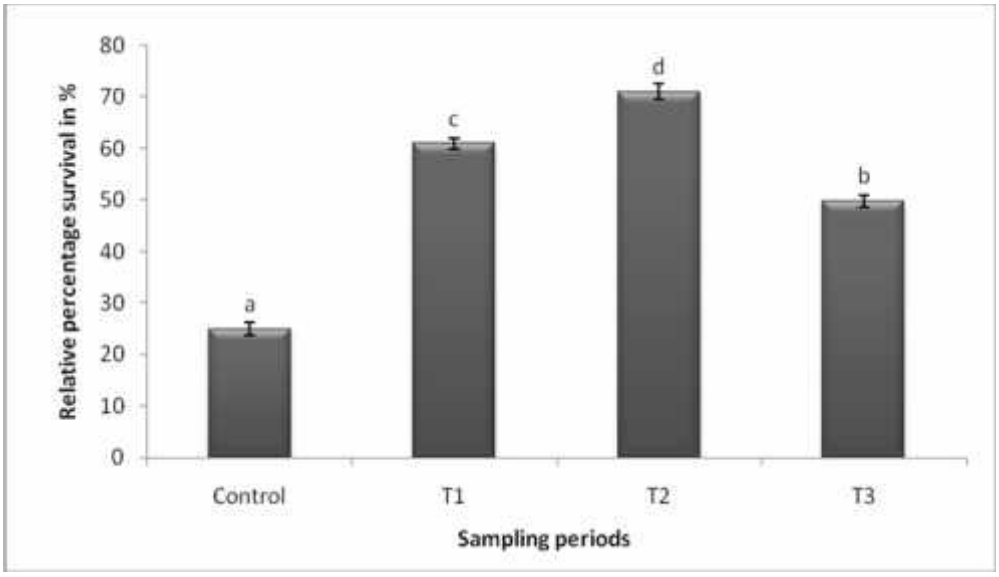

Fig. 5. Relative percentage survival (RPS) in *L. rohita* differently fed with 0% (control), 1%, 2% and 3% of *M. cordata* (values are mean ± SE). a,b,c,d different letters indicate significant difference between different treatment groups within a sampling day ($p < 0.05$) (n= 60 fish/replicate).

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