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Effect of *Azadirachta indica* Leaf Soluble Fraction on Immune Response and Disease Resistance in *Channa striatus* Against Tropical Freshwater Fungal Parasite *Aphanomyces invadans* (EUS)

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Abstract: *Aphanomyces invadans,* a fungal parasite is the main causative agent for Epizootic Ulcerative Syndrome (EUS) that causes series damage to tropical freshwater murrel (*Channa striatus*). *Azadirachta indica* to develop a non-specific humoral responses and disease resistance against *A. invadans* were tested. The extracts of *A. indica* were administrated to *A. invadans* infected *C. striatus,* after 1 to 4 weeks of experimental period. Fish were intraperitoneally injected with 0, 2, 20 or 200 mg kg^{-1} body weight, of the hexane soluble fraction. The present study determined the effect of hexane soluble fractions of *Azadirachta indica* on the hematological, biochemical, immunological responses and histology in *C. striatus.* All the soluble fractions exhibited inhibitory zones higher than the antibiotic Ampicillin against bacterial and fungal pathogens. The organic solvent extract from hexane soluble fraction showed a high inhibition zone. The red blood cells (RBCs), white blood cells (WBCs), hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly increased in murrel with *A. indica* soluble fractions.. The serum lysozyme activity, phagocytic activity, Total protein content was significantly enhanced in a dose of 20 mg kg^{-1} of hexane soluble fractions administrated groups on the weeks 2 to 4. The alternative complement activity was found to be increased in hexane fraction administered groups. The present results indicated that *A. indica* soluble fraction at a dose of 20 mg kg^{-1} significantly enhances the immunological parameters and improves the innate immune system in *C. striatus* against *A. invadans.*

Keywords: *Azadirachta indica* · *Channa striatus* · *Aphanomyces invadans* · Immune Response · Fungal Parasites

INTRODUCTION

Aphanomyces invadans is the main aetiological component of a serious disease of Indian freshwater and marine fish known as epizootic ulcerative syndrome (EUS). A diverse group of biotic agents such as viruses, bacteria and cutaneous ectoparasites may initiate skin lesions, which are subsequently colonized by *A. invadans* and ultimately lead to EUS. The EUS has been reported from 24 countries in four continents and more than 100 fish species have been affected by EUS [1]. The EUS spreads in a fish culture pond during the periods of low

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temperatures or 18-22°C and after periods of heavy rainfall. The striped snakehead, *Channa striatus* is locally known as striped murrel was one among the highly priced air breathing freshwater fishes and is highly regarded as food fish in the South and Southeast Asian countries. They are often affected by the dreadful disease Epizootic Ulcerative Syndrome (EUS) and encounter heavy losses in capture as well as culture fisheries in southern region of India particularly, Cauvery and Bhavani River basins.

Herbal drugs are known to possess immune-modulatory properties and generally act by stimulating both specific and non-specific immunities against EUS disease in fishes. Many plants used in traditional medicine are reported to have immune-stimulating properties. The traditional measures of prevention and control of fish diseases includes the application of antibiotics and vaccines. Recently attention has been focused on *A. indica* leaf extracts, it has been used to protect the blood parameters and increase immunity in fish against *A. invadans* [2]. The neem (*A. indica*) is perhaps the most useful traditional medicinal plant in India. The chemical constituent includes alkaloids, flavonoids, triterpenoids, phenolic compounds, Carotenoids, steroids and ketones. Azadirachtin is a mixture of seven isomeric compounds labeled as azadirachtin A-G, among this is more effective [3]. Previous reports also states the presence of non-isoprenoids, amino acids, polysaccharides, polyphenolics (Flavonoids) were reported in neem leaf [4]. Different parts of *A. indica* plant (leaves, bark, fruit, flowers, oil and gum) were documented to be associated with various remedial properties such as antimicrobial effects [5], hepatoprotective action [6], neuroprotective effect [7], invitro antiviral [8], insecticidal [9] and acaricidal [10].

A number of herbs and its products have been tested for enhancing growth, non-specific and specific immune system in finfish and shell fish [11]. The herbal immunostimulants which have been tested includes *Ocimum sanctum* extracts [12], azadirachtin [13] *Radix astragali seu Hedysari* and *Radix angelicae sinensis* [14] *Cynodon dactylon*, *Piper longum*, *Phyllanthus niruri*, *Tridax procumbens*, *Zingiber of?cinalis* [15], *Styrax japonica* and *Solanum nigrum* [11], *Nyctanthes arbortristis* [16], *Tinospora cordifolia* [17] and *Magnifera indica* [18] extracts. Hence, the aim of the present study is to improve the hematology, specific and nonspecific immune response in snakehead fish *C. striatus,* by administering hexane soluble fractions of *A. indica* leaves intraperitoneally against *A. invadans* (Epizootic Ulcerative Syndrome).

MATERIALS AND METHODS

Experimental Animal Collection: The diseased murrel (*C. striatus*) with average length of 20cm and 180g of weight were collected during monsoon season from a Saravana fish farm Kaveripatti, Namakkal District, Tamilnadu, India. Their health status was examined immediately upon arrival. The murrel were reared in cement tanks (6m×2m×2m) and fed with semi moist formulated feed (Fig. 1).

Isolation of *Aphanomyces Invadans:* The *A. invadans* was isolated from *C. striatus* based on the external symptoms (Unresponsiveness, wound infection, irregular pattern, superficial lesions, swelling discoloration and deep ulcer hemorrhages). *A. invadans* species were identified according to their morphology using potato dextrose agar medium. The culture was routinely maintained in glucose-peptone-penicillin-oxolinic acid broth for 3-4 weeks at room temperature and sub-cultured on GP-POX agar for 5 days. These broths were used for further antimicrobial studies.

Preparation of Leaf Extract: The neem plant (*A. indica*) leaves were collected from residential premises nearby Bharathiar University, Coimbatore, India. The plant was authenticated by Plant Taxonomist, Botanical Survey of India, Tamil Nadu, Agriculture University, Coimbatore, Tamil Nadu, India. The fresh leaves were washed in sterile distilled water, dried in shade, powdered and stored at -20°C. The extraction was carried out by following as described by Xu *et al.* [19]. All the extracts were tested for their antibacterial and antifungal activity.

Fig. 1: Aphanomyces invadans infected Channa striatus fish

Antibacterial Activity of *A. Indica* Extract: The antibacterial activity of *A. indica* hexane soluble fractions were determined against 5 bacterial strains of *Yersinia ruckeri*, *Aeromonas hydrophila, Aeromonas formican, Aeromonas liquefaciens* and *Pseudomonas aeruginosa* (IMTECH, Chandigarh, India). Antimicrobial activity was measured using the standard diffusion disc plate assay. The antimicrobial activity was determined by observing the zones of suppression of bacterial growth around the 3 mm diameter well and measured in millimeter.

Antifungal Activity of *A. Indica* Extract: The antifungal activity of hexane soluble fractions of *A. indica* was determined against 5 fungal strains of *A. invadans, Aspergillus niger, Aspergillus flavus, Candida albicans and Cryptococcus neoformans* (IMTECH, Chandigarh, India). The hexane soluble fractions was carefully sprayed on 6 mm Whatman No1 disc filter paper and placed on culture medium and incubated at 28°C. Inhibition zones were measured after 24 hours of incubation. Standard antibiotic Ampicillin (10 mcg) disc was used as control.

Experimental Regime: The experimental fishes were divided in to five groups. In each experimental tank a total of ten *C. striatus* fishes and fed with semi moist formulated feed.

Administration of Extract in Fishes: To study the non-specific immune mechanisms, fishes were intraperitoneally injected with 0.2 ml of hexane soluble fraction of *A. indica* leaves at the dosage of Control, 0.2, 2.0, 20 and 200 mg kg^{-1} body weight using 1 ml tuberculin syringe with 24-gauge needle on day 1. The control fish received 0.2 ml of sterile distilled water. The fishes were bled 2 days prior to experiment and 2, 4, 6, 8 and 10 days after treatment. To study the disease resistance of fishes, hexane soluble fraction of *A. indica* leaf administered intraperitoneally as double dose on Day 1 and 4.

Hematological Analysis: The blood was collected into vacuum tubes containing heparin as anticoagulant (Greiner). The levels of RBCs and WBCs were counted by hemocytometer, Hb concentrations were estimated by Cyanomethaemoglobin method [20] and Hct was MCV, MCH and MCHC were also calculated using standard formula.

$$\text{MCV (cubic micron)} = \frac{\text{Hct (\%)}}{\text{RBC (millions} \times \text{cu mm} \times 106)} \times 100$$

$$\text{MCH (pictograms)} = \frac{\text{Hb (g/dl)}}{\text{RBC (millions} \times \text{cu mm} \times 106)} \times 100$$

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl)}}{\text{Hct (\%)}} \times 100$$

Immunological Assays: The phagocytic activity of macrophages was determined by the method of Sakai *et al.* [22]. Alternative complement activity was examined following the procedure of Yano [23] using rabbit red blood cells. The lysozyme activity was determined by turbidimetric assay following Parry *et al.* [24]. The Total protein content in Serum was estimated by employing Folin-Ciocalteau reagent method described by Lowry *et al.* [25].

Histological Analysis: The *A. invadans* affected experimental fish tissue pathology was examined based on histological studies. Fish muscles were surgically removed and fixed in Bouin's fixative and analyzed using conventional histolopathological techniques. Analytical samples of 6µm were sectioned out and stained with haemotoxylin and eosin stains [26].

Statistical Analysis: Values of each parameter were measured and expressed as the Arithmetic Mean ± Standard Deviation (SD). The effect of *A. indica* soluble fractions on hematological, biochemical and immunological parameters of experimental fishes were tested using one-way ANOVA and a comparison of the mean values was done using Duncan's multiple range tests at 0.05% level of significance using the software program SPSS (Version 14.0; SPSS) for Windows was used for the analysis.

RESULTS

Antibacterial Activity: The *A. indica* soluble fractions A (Distilled water), B (Hexane) and C (Ethanol) showed a higher level of restriction zone against all fish bacterial pathogens strains when compared to antibiotic Ampicillin. It was statistically significant for both treatment and bacterial strains at 0.01% level and between treatment and bacterial strains it also showed significance at 0.05% levels. Hexane soluble fractions showed a higher inhibition zone against all fish bacterial pathogens than commercial antibiotics (Fig. 2).

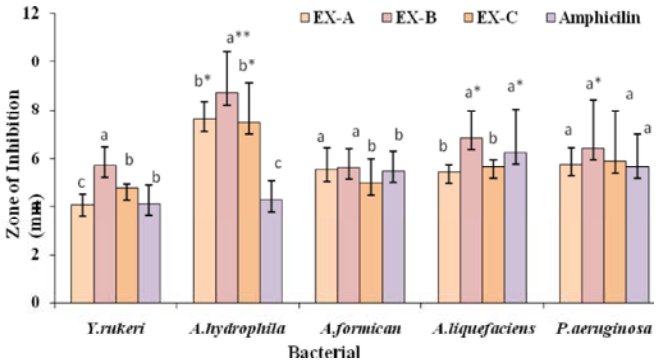


Fig. 2: The Antimicrobial activity of *A. indica* different soluble fractions against bacterial pathogens. **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly (*P<0.05*).

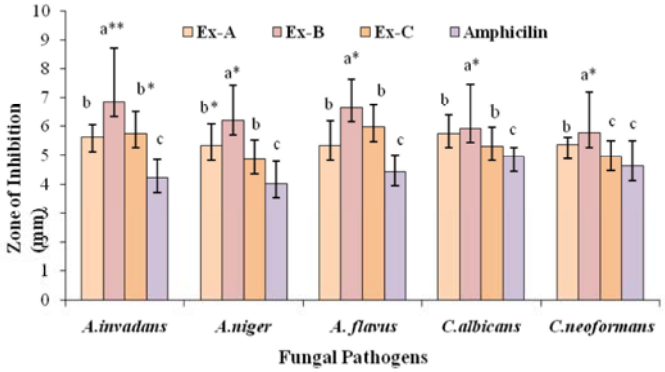


Fig. 3: The Antimicrobial activity of *A. indica* different soluble fractions against fungal pathogens. **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly (*P<0.05*).

Antifungal Activity: The antifungal activity of different soluble fractions (A-C) of *A. indica* leaves was determined against five fungal pathogens. The neem soluble fractions showed a higher level of restriction zone against fungal colonies when compared to antibiotic Ampicillin. The *A. indica* hexane soluble fraction showed restriction zone inhibition on fungal pathogen culture plates than the commercial antibiotics. The data was statistically significant for both treatment and fungal strains at 0.01% level (Fig.3).

Hematology: The *A. indica* hexane soluble fractions were intraperitoneally administered at different doses to stimulate blood from week 1 to 4. When compared to the 0 mg kg^{-1} administered *C. striatus,* the RBCs level (10^6 mm^{-3}) was significantly increased from week 4 of 20 mg kg^{-1}compared to 0 mg kg^{-1}, 0.2 mg kg^{-1}, 2.0 mg kg^{-1} and 200 mg kg^{-1} respectively. The WBCs count was gradually increased from week 1 to week 4. White blood cells (WBCs), hematocrit (Hct) and hemoglobin (Hb) significantly increased in *A. invadans* infected fish administered with neem soluble fractions against fungal pathogen from week 1 to 4. However, fishes treated with neem extract group significantly showed no change in WBCs, Ht and Hb. In the present study, 20 mg kg^{-1} administered group showed better elevation of these blood parameters such as RBCs, WBCs, Ht and Hb. The mean corpuscular volume MCV was significantly increased in infected murrel fishes treated with hexane extract against fungal pathogen from weeks 1 to 4. The MCH (pg) and MCHC (%) value was increased (Except in 1st week) significantly (*p<0.05*) in *A. indica* hexane soluble fractions administered fishes throughout the experimental study period (Table 1).

Lysozyme Activity: The serum lysozyme activity was significantly enhanced by the administration of *A. indica* 20 mg kg^{-1} of hexane soluble fractions on weeks 2 to 4. However, the serum lysozyme activity significantly enhanced *A. invadans* affected *C. striatus* with *A. indica* soluble fractions from weeks 2 to 4 compared to Control fish (Fig. 4).

Table 1: Changes in hematological parameters of *C. striatus* (mean±SD, n=5) administered with 0, 0.2, 2.0, 20 and 200 mg/kg^{-1} *A. indica* hexane soluble fractions (B) against *A. invadans*.

Hematological Parameters	Doses (Mg/kg)	Week 1	Week 2	Week 3	Week 4
RBC(10^6 mm^{-3})	0	1.22±0.16^b	1.32±0.31^c	1.43±0.35^c	2.10±0.54^c
	0.2	1.36±0.18^b	1.56±0.18^b	1.74±0.37^b	2.28±0.37^b
	2.0	1.65±0.32^b	1.77±0.01^b	1.90±0.47^b	2.42±0.35^b
	20	2.43±0.47^a	2.57±0.33^a	3.19±0.25^a	3.88±0.23^a
	200	2.82±0.34^a	2.88±0.65^a	2.97±0.69^a	2.68±0.65^b
WBC (10^4mm^{-3})	0	122.6±1.53^c	134.3±3.16^b	137.2±2..95^a	138.3±1.74^c
	0.2	124.8±1.92^c	130.1±1.20^b	130.7±3.62^a	145.0±2.00^b
	2.0	126.6±1.30^b	130.8±3.34^b	135.6±5.62^a	146.8±3.78^b
	20	128.8±1.43^a	136.4±2.07^a	140.6±6.14^a	155.8±4.27^a
	200	126.6±2.30^b	134.0±2.44^b	137.1±3.00^a	146.6±6.99^b
Hematocrit (%)	0	11.38±1.35^b	11.41±1.37^c	14.28±5.98^b	19.28±3.63^c
	0.2	12.11±1.60^b	12.39±1.26^b	14.57±5.32^b	21.83±2.43^b
	2.0	12.07±2.80^b	12.55±3.58^b	15.35±4.22^a	20.63±3.19^b
	20	16.73±3.44^a	14.68±3.67^a	15.44±7.13^a	24.22±1.68^a
	200	12.49±1.89^b	12.88±1.50^b	14.77±3.13^c	23.62±2.40^a
Hemoglobin (g dl^{-1})	0	4.35±0.13^a	4.42±2.19^c	5.12±3.37^c	5.28±1.57^c
	0.2	4.63±0.23^a	4.76±2.25^b	5.20±3.51^b	5.33±3.38^c
	2.0	4.64±0.20^a	4.88±1.74^b	5.37±2.42^b	6.04±4.05^b
	20	4.72±0.30^a	5.20±1.62^a	6.28±1.39^a	7.23±1.46^a
	200	4.67±0.20^a	4.91±1.98^a	5.21±1.16^b	6.92±1.10^a
MCV(fl)	0	47.0±3.87^b	48.4±2.77^c	49.6±1.34^c	51.8±2.48^c
	0.2	48.5±2.30^b	52.6±3.40^b	53.2±2.16^c	53.8±3.61^c
	2.0	51.7±1.33^a	56.4±1.45^b	57.4±3.64^b	69.0±4.09^b
	20	60.2±0.58^a	63.1±3.10^a	66.3±1.51^a	70.23±1.58^a
	200	48.6±3.04^b	64.9±1.56^a	61.4±1.74^b	64.81±2.69^c
MCH (pg)	0	7.4±2.30^c	16.0±1.58^c	16.2±1.30^c	16.4±0.89^c
	0.2	7.4±1.89^c	17.6±0.89^c	16.8±1.92^c	19.4±1.51^c
	2.0	9.6±1.89^b	22.0±0.70^b	24.0±2.73^b	23.6±1.14^b
	20	14.4±1.70^a	27.2±2.48^a	27.6±0.89^a	30.4±0.8^a
	200	15.0±2.87^a	23.4±1.34^b	25.2±2.28^b	25.0±3.24^b
MCHC (%)	0	9.65±1.25^c	9.95±1.86^c	10.65±2.43^c	21.23±1.26^b
	0.2	10.68±1.89^c	11.71±2.83^b	11.29±2.83^c	20.8±2.63^c
	2.0	11.63±1.78^b	12.56±2.42^b	12.86±3.54^b	22.92±3.85^b
	20	13.49±2.07^a	14.53±1.61^a	15.02±1.46^a	25.98±2.74^a
	200	11.66±1.32^b	12.79±1.79^b	13.02±1.05^b	23.35±1.87^b

Mean ± SD (n=5) Mean values within the same row sharing the same superscript are Significant different ($P > 0.05$).

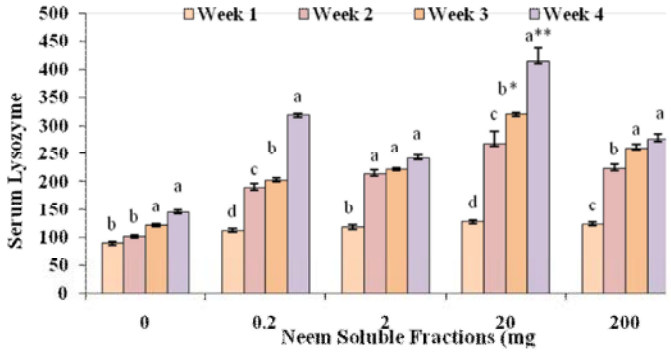


Fig. 4: The Lysozyme activity in *C. striatus* administered with different doses of *A. indica* soluble fractions (B) against *A. invadans*. **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly ($P<0.05$).

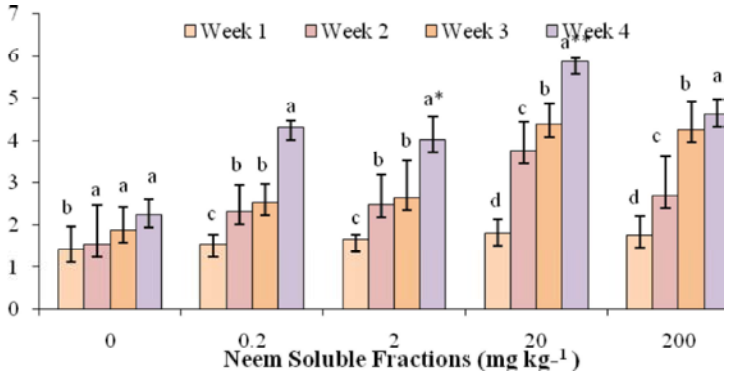


Fig. 5: The Total Protein content in *C. striatus* administered with different doses of *A. indica* soluble fractions (B) against *A. invadans* **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly (*P<0.05*).

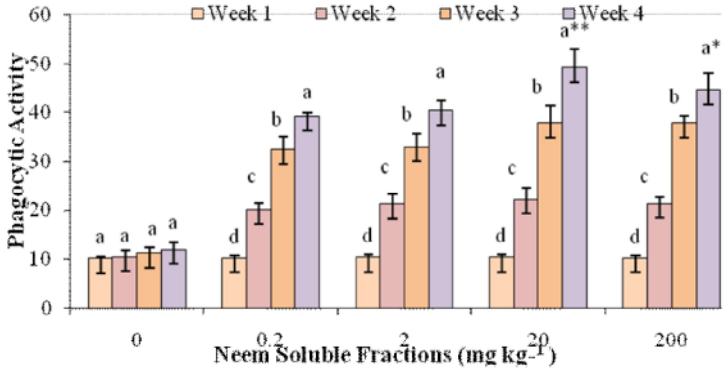


Fig. 6: The Phagocytic activity in *C. striatus* administered with different doses of *A. indica* soluble fractions (B) against *A. invadans*. **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly (*P<0.05*).

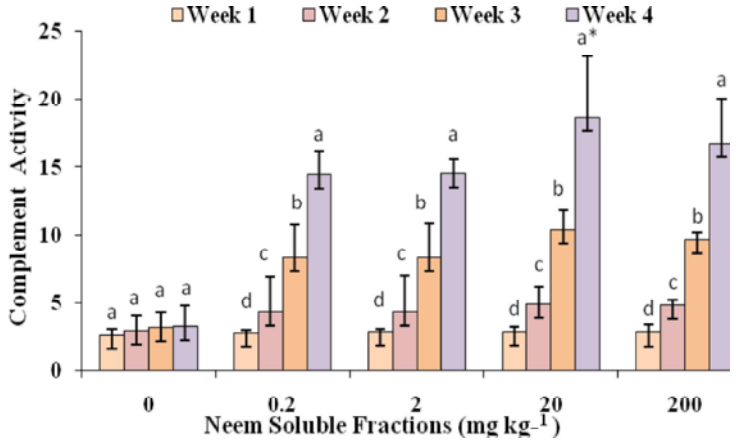
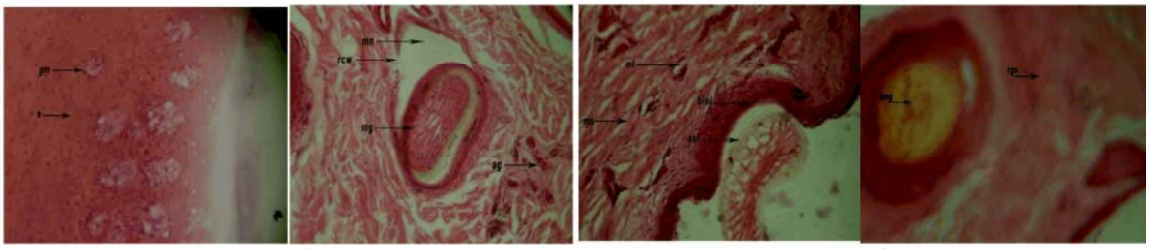


Fig. 7: The Complement activity in *C. stiatus* administered with different doses of *A. indica* soluble fractions (B) against *A. invadans* **Significant at 0.01 level; *Significant at 0.05 level. Mean in a bar followed by a different letters are significantly (*P<0.05*).



8a 8b 8c 8d

Fig. 8: Histopathological photomicrograph of *A.invadans* infected *C.striatus* muscle epidermal layer. 8a. 3rd Day of Zoospores administered *C. striatus* muscle histology. 8b.7th Day of matured Zoospores 8c. 10 Day of Zoospores administered *C. striatus* muscle releasing of spores through epidermis 8d. *A. indica* soluble fractions administered *C. striatus* muscle histology gm- granuloma, mg- mycotic granuloma, mn- myonecrosis, hl- haemorrigic lesions, rcw- regenarating cells from wound, mt- myotome, asf- *A.invadans* spore formation, s- superficial lesions, mtia- multinucleate formations of injected areas, sg - small granuloma, ms- muscle shrinkage, hlel- hemorrhagic lesions with epidermal layers, dmg- damaged mycotic granuloma smn - shrinked myconecrosis, rgn - rearranged muscles

Total Protein: The total protein levels of experimental fish serum were significantly increased in *A. invadans* infected fish intraperitoneally administered with 20 mg kg^{-1} (5.86±0.10 mg/ml^{-1}) hexane soluble fractions of *A. indica* from weeks 1 to 4 (Fig. 5) when compared to the control groups, all the statistical values were significant at p <0.05.

Phagocytic Activity: The phagocytic activity of the snakehead fish kidney macrophages increased significantly in fishes treated with neem hexane soluble fractions (Fig.6). However, no significant phagocytic activity had been found in groups which were not treated with hexane soluble fractions in diseased *C. striatus* from week 1 to 4 when compared to control with 0.2 to 200 mg kg^{-1}. All values were statistically significance at p<0.05.

Alternative Complementary Activity: Alternative complementary activity was observed from fishes intraperitoneally administered with 0.2, 2.0, 20 and 200 mg kg^{-1} neem plant extract but not with control from weeks 1 to 4. The alternative complementary activity was significantly enhanced in fishes treated with 20 mg kg^{-1} (Fig.7).

Histology: The fish had severe swollen hemorrhagic lesions and the tissue pathology of the *C. striatus* fish was typical in disease condition showing massive proliferation of hyphae in the lesion area and out of the epidermic layer deeply penetrating the ulcer (8a-c). These extensive pathological changes were always associated with gross visible hemorrhagic lesions and morbidity. Normal muscle structure was observed in most of the areas after administration of *A. indica* and this shown pronounced activity. Importantly, treatment with soluble fractions prevented both *A. invadans* induced necrosis and inflammation. The degree of muscle structure also decreased in *A. indica* treated *C. striatus*. Consistent with the improved histology, the lack of mycotic necrosis, inflammation, damaged the granulomas and regeneration of muscles (Fig.8d). *A. indica* soluble fractions treated groups shown curative effect to some extent in tissues levels.

DISCUSSION

The outbreak of EUS was found to be associated with low and declining water temperatures and high rainfall [27]. Snakeheads have been repeatedly described as one of the most EUS susceptible species [28]. In the present study, *A. invadans* infected fresh water murrel *C. striatus* after intraperitoneally administered with different concentrations of *A. indica* hexane soluble fractions showed significant weight gain compared to infected fish without soluble fractions administration. Recently commercial fisheries focus their attention on the use of natural immunostimulants for the enhancement of non-specific immunity to counter disease outbreaks and it is increased the hematological parameters of *A. hydrophila* infected *C. punctatus* [29]. In the present study Hb and Hct level did not significantly change with any diet on first week; it significantly increased by the 4th

week when fed with 20 mg kg^{-1} of *A. indica* injected fishes that are challenged with *A. invadans*. The present study also identified a significantly low in RBCs and Hb contents in 0mg/kg group compared to other experimental groups, is possibly due to hypochromic microcytic anemia caused by the parasites. The RBCs abnormalities such as, viral inclusions, hemoglobin cysts and hemoparasites are linked to nutritional status [30].

The decreased RBCs counts, Hct and Hb concentrations indicate that RBCs are being destroyed by the leucocytosis activity leading to erythrocytic anemia [31]. For instance, the pearl spot *Etroplus suratensis* infected with EUS becomes aneamic and subsequently suffered a significant reduction in RBCs, Hb and PCV levels [32]. Similarly, *Allium sativum* influenced erythrocyte, leucocyte and Ht content in *Piaractus mesopotamicus* against *Anacanthorus penilabiatus* [30]. The MCV, MCH and MCHC significantly decreased in the fish were injected with 0mg/kg group, decrease in MCV may be attributed to the swelling of the erythrocytes resulting in Amacrocytic aneamia or Macrocytic anaemia in ?shes exposed to stress [33]. The results are in agreement with *L. rohita* and *Oncorhynchus mykiss* after dietary administration with *A. aspera* and garlic against *A. hydrophila* infection [34].

In the present study lysozyme activity was well enhanced on treatment with methanolic and water soluble fractions of *A. indica*. Similar observations of increased values of fish serum lysozyme activity was reported to be enhanced on treatment with water or hexane soluble fractions of *S. trilobatum* leaves in *O. mossambicus* and *A. aspera* seed in *L. rohita* [34]. Chinese herbs in *Carassius auratus* [35] enhanced the lysozyme activity. Total protein level significantly increased in *C. striatus* with 20 mg kg^{-1} neem fractions intraperitoneally administrated group which were considered to reflect strong innate immunity in fish [36-37].

The immunostimulants mainly facilitate the function of phagocytic cells, increase their bactericidal activities and stimulate the natural killer cells, complement system, lysozyme activity and antibody responses in fish and shellfish which confer enhanced protection from infectious diseases [11]. Complement, is one of the most important serum factors because of its activating effects on the cellular defenses. In the present, the complement activity was significantly enhanced on intraperitoneally administration of hexane soluble fraction of *A. indica.* Similar results were reported in juvenile channel catfish [38] and Atlantic salmon [39] resulted in increased complement activity.

Common carp, *Cyprinus carpio* L. inoculated with *A. piscicida* showed no gross signs of inflammation and mycotic lesions occurred only around the injection site [40]. Neem paste treated individuals showed complete wound healing on the 6th day of the treatment and the aloe paste treated murrel showed slower recovery in the 8th day of treatment [41]. In conclusion, the present study confirmed that *A. indica* extract at 20mg kg^{-1} doses act as immunostimulant and has a positive effect of *A. invadans* fungal disease resistance and primary immune response.

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