Immunostimulatory effect and disease resistance induced by *Lawsonia inermis* against *Aphanomyces invadans* in striped murrels (*Channa striatus*)

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ABSTRACT

*Aphanomyces invadans*, the main causative agent for Epizootic Ulcerative Syndrome (EUS) causes series damage to freshwater fishes particularly striped murrels (*Channa striatus*). The present study determined the effect of methanolic soluble fractions of *Lawsonia inermis* on hematology and immune response in *C. striatus*. The extracts of *L. inermis* were administrated to *A. invadans* infected *C. striatus*, after 1, 2 to 4 weeks of infection. Red blood cells (RBC) significantly increased in murrels with *L. inermis* soluble fractions. White blood cells (WBC), haematocrit (Ht) and hemoglobin (Hb) significantly increased compared to Control (untreated). However, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) significantly decreased. The serum lysozyme activity was significantly enhanced in 60 mg/kg¹ methanolic soluble fractions administrations groups on weeks 2 and 4. All the soluble fractions exhibited inhibitory zone than antibiotic Ampicillin against bacterial and fungal pathogens. Organic solvent extract from Methanol soluble fraction showed high inhibition zone. The Hemolytic activity increased with methanolic soluble fractions. Histological observations illustrated a gradual repair of damaged muscles necrosis, macrophage infiltration, fibrosis and mycotic granulomas caused by *A. invadans*, when treated with *L. inermis*. The present results indicate that *L. inermis* soluble fraction at 60 mg/kg¹ levels significantly enhance the immunological and histological parameters, and enhance the innate immune system in murrels *C. striatus* against *A. invadans*.

**Keywords**: *Lawsonia inermis*, *Channa striatus*, Epizootic Ulcerative Syndrome, *Aphanomyces invadans*, Hematology, Immunostimulant.
1. INTRODUCTION

The single most important drawback of large-scale commercial culture of several fish species is the deficiency of quality seed of uniform size, and free of diseases, parasites and pests at the time of stocking in culture ponds [1]. Over the past 2 decades, epizootic ulcerative syndrome (EUS) had a serious impact on tropical fisheries resulting in heavy economic loss [2]. EUS has been reported from 24 countries on four continents and more than 100 fish species have been affected by EUS [3]. A diverse group of biotic agents such as viruses, bacteria, and cutaneous ectoparasites may initiate skin lesions, which are subsequently colonized by Aphanomyces invadans and ultimately lead to EUS [4]. Aphanomyces invadans-induced epizootics are characterized grossly by the development of deeply penetrating ulcers and microscopically by extensive myonecrosis and granulomatous myositis [5].

EUS occurs mostly during periods of low temperatures and after periods of heavy rainfall. The striped snakehead, Channa striatus one among the highly priced air breathing freshwater fishes and a good table fish in the Southeast Asian countries. They are often affected by the dreadful disease Epizootic Ulcerative Syndrome (EUS) and encounter losses in capture as well as culture fisheries particularly in the southern region of India, at Cauvery and Bhavani river basins.

Indian fish farmers so far followed antibiotics and vaccines application to prevent the infection of EUS, which was not success. Recently attention has been focused on immunostimulants and plant products which could have a beneficial effect in fish disease management. A number of herbs and its products have been tested for enhancing growth, non-specific, and specific immune system in fin fish and shell fish [6]. The herbal immunostimulants which have been tested includes saponin [7], glycyrrhizin [8], aloe vera [9]. Ocimum sanctum extracts [10] azadirachtin [11]. Oroglo layer dry, a xanthophyll preparation from Marigolds [12], Viscum album, Urtica dioica and Zingiber officinalis [13]. Radix astragalin seu Hedysarum and Radix angelicae sinensis [14], Astragalus radix and Scutellari radix [15] and Achyranthes aspera [16] Solanum trilobatum [17] and Eclipta alba [18]. Cynodon dactylon, Piper longum, Phyllanthus niruri, Tridax procumbens, and Zingiber officinalis[19], Styrax japonica [6], Solanum nigrum [6]. Nyctanthes arbor-tristis [20] Tinospora cordifolia [21] and Magnifera indica [22] extracts.

Lawsonia is monotypic genus, represented by Linerms, native of North Africa and south-west Asia, widely cultivated as an ornamental and dye plant throughout India [23]. This plant is commonly known as Henna or Mhendi and abundantly available in tropical and subtropical areas. Ancient history of India describes its diverse uses and also plays appreciable role in Ayurvedic or natural herbal medicines [24]. The leaves of L. inermis also reported to contain soluble matter tannin, gallic acid, glucose, mannitol, fat, resin and mucilage [25]. Traditionally used in cosmetics, the leaf dye of L. inermis is used to stain hands, feet and nails with artistic patterns. However, L. inermis has also been used in ethno-medicine to treat various maladies including, but not limited to, arthritis, headaches, ulcers, diarrhea, leprosy, intestinal neo-plasticity, jaundice, fever, leucorrhoea, diabetes, and smallpox [26]. It is used as medicinal plant because of its attributed antibacterial, antifungal, anti-amoebiasis, astringent, anti-hemorrhagic, hypotensive and sedative effect [27]. There is evidence of the plant having wound healing properties [28]. Furthermore, treatment with hydro-alcoholic extract of L. inermis (in vivo) has been proved to increase levels of cellular antioxidant enzymes such as glutathione reductase, superoxide dismutase and catalase [29].

The aim of the present study was to evaluate the haematology, specific and nonspecific immune response in snakehead fish Channa striatus, intraperitoneally administered Linerms leaf extract and methanolic soluble fractions against Aphanomyces invadans (Epizootic Ulcerative Syndrome).

2. MATERIALS AND METHODS

2.1. Experimental Animals

Diseased murrels (C. striatus) with average length 20cm and weight 180g were collected during monsoon season from a private fish farm Kaveripatti, Namakkal District, Tamilnadu, India. Their health status was examined immediately upon arrival. The manually EUS infected fish were separated and transported to laboratory. They are identified by the lesions and deformed fins at the dorsal side and showed reddish spots on the ventral side. These diseased murrels were reared in cement tanks (3m×1m×1m) and fed with semi moist formulated feed.
2.2. Isolation of A. invadans

A. invadans was isolated from C. striatus which manifested it and showed external symptoms (unresponsiveness, wound infection, irregular pattern, superficial lesions, swelling discoloration, and deep ulcer hemorrhages). A. invadans species were identified according to their morphology by culturing in potato dextrose agar. The culture was routinely maintained in glucose-peptone-phenicol-ofoxolin acid broth (GP-POX broth; for 3-4 weeks at room temperature and sub-cultured on GP-POX agar for 5 days. This broth was used for further antimicrobial studies.

2.3. Preparation of leaf extract

Henna Plant leaves (L. inermis) were collected from residential premises nearby Bharathiar University, Coimbatore. 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 done following the methods of [30,31]. Leaf powder was extracted with methanol and was filtered through sterile muslin cloth. The filtrate was centrifuged at 4000 RPM for 10 min and the solvent was evaporated using rotary vacuum evaporator. The residue obtained after evaporation was mixed with sterile distilled water. The extract was taken in a separating funnel with equal volume of methanol and mixed carefully by intermittently releasing the pressure. The content was allowed to stand without any disturbance until two distinct layers; lower water soluble and upper methanol soluble ones were seen. The fractions were collected separately. This process was repeated till the colourless methanol fraction was obtained indicating the completion of fractionation. The methanol soluble fraction was concentrated in rotary vacuum evaporator and stored at 20°C. All the extracts were tested for their immunomodulatory activity.

2.4. Antifungal activity of L. inermis extract

Antifungal activity of L. inermis leaf extract was dissolved at 5% in - A (Distilled water), B (Ethanol) and C (Methanol). The antifungal activity was determined against A.invadans (EUS) using the standard diffusion disc plate assay [32]. 5% of diluted soluble fractions was carefully sprayed on 6 mm whatman No1 disc filter paper and placed on A. invadans, Aspergillus niger, Aspergillus flavus and Candida albicans 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.

2.5. Hematological and Biochemical analysis

The fish blood was collected by vein puncture, and transferred into vacuum tubes containing heparin as anticoagulant (Greiner). RBC and WBC were counted by haemocytometer method [33], Hemoglobin (Hb) concentrations were estimated by Cyanomethaemoglobin method [34] and Hct was determined by the micro-hematocrit method [35]. Erythrocyte indices like MCV, MCH and MCHC were also calculated according to standard formulas [36]. The Total protein content in Serum was estimated by employing Folin-Ciocalteau reagent method of Lowry et al. [36].

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\frac{\text{MCV (cubic micron)}}{\text{RBC (millions \times cu mm \times 10^6)}} \times 100
\]

\[
\frac{\text{MCH (pictograms)}}{\text{RBC (millions \times cu mm \times 10^6)}} \times 100
\]

\[
\frac{\text{MCHC (g/dl)}}{\text{Hct (\%)} \times 100}
\]

2.6. Extract administration in fish

To study the non-specific immune mechanisms, fish were intraperitoneally injected with 0.2 ml of methanolic soluble fraction of L. inermis leaves at the dosage of 0 (Control), 0.6, 6, 60 and 600 mg kg⁻¹ body weight using 1 ml tuberculin syringe with 24-gauge needle on day 0. The control fish received 0.2 ml of sterile distilled water. The fish were bled 2 days prior to and 2, 4, 6, 8 and 10 days after treatment. To study the disease resistance of fish, methanolic soluble fraction of L. inermis leaf administered intraperitoneally as double dose on Day 1 and 4.

2.7. Lysozyme activity

Lysozyme activity was measured by the method of Parry et al. [37], in combination with the microplate adaptation of Hutchinson and Manning,[38]. In this turbidimetric assay, 0.03% lyophilized Micrococcus lysodeikticus in 0.05mM Sodium phosphate buffer (pH 6.2) was used as substrate. Ten micro-litres of fish serum was added to 250 ml of bacterial suspension in a “U” bottom microtitre plate and the reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min incubation at 22°C using a
microplate reader (Bio-Rad, USA). One unit of Lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

2.8. Haemolytic Complement Activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [39]. Aliquots (500 µl) of test serum as complement source, serially diluted in Hank’s buffer (HBSS), were added to 500 µl of SRBC and cow red blood cells (CRBC) (final concentrations 10-0.078%). After incubation for 1 hr. at 22°C, the samples were centrifuged (800 x g for 5 min at 4°C) to remove non-lysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 540 nm in a spectrophotometer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 500 µl of distilled water or HBSS to 500 µl samples of SRBC and CRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting Y/ (1-Y) against the volume of serum added (ml) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH50) was determined and the number of ACH50 unit/ml was obtained for each experimental group.

2.9. Statistical analysis

Values of each parameter measured and were expressed as the arithmetic Mean ± Standard Deviation (SD). Effects of *L. inermis* administrated fish hematological, biochemical and immunological 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.

3. RESULTS AND DISCUSSION

In the present study, methanolic soluble fractions of *L. inermis* leaves were found to be significant stimulatory effect on the specific, nonspecific immunological response against *A. invadans* disease resistance in *C. striatus*. In the recent years, there is increasing interest in the use of herbal extracts as dietary and therapeutic supplements indicate that modulate immune function in fish and shell fish [40, 41]. Antifungal activity of *L. inermis* leaves different soluble fractions - A (Distilled water), B (Ethanol) and C (Methanol) were determined against *A.invadans* (EUS). Soluble fractions showed a higher level of restriction zone against fungal colonies when compared to antibiotics Ampicillin (Figure 1). It was statistically significant for both treatment and fungal strains at 0.01% level. *L. inermis* extracts showed an excellent inhibition zone (particularly methanolic soluble fractions) against fish pathogens than the antibiotic Ampicillin. Several reports indicated that *S. nigrum* exhibited anti-ulcer, antitumor, anti-oxidant properties [42] and significantly increased the hematological parameters of *A. hydrophila* infected *C. punctatus* [43]. *A.invadans* infected *C.striatus* with *L.inermis* soluble fractions intraperitoneally administrated at different doses to stimulate specific and nonspecific immune response from weeks 1 to 4 when compared to the 0 mg/kg⁻¹ (Control). However, RBC was significantly increased from weeks 1 to 4 of 60 mg/kg⁻¹. The WBC, Hb and Ht percentage levels significantly increased in *A.invadans* infected fish with all doses of *L. inermis* extracts administrated from weeks 1 to 4 as compared to 0 mg/kg⁻¹ (Control). The mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) did not change significantly with any supplementation diet on weeks 1. However, MCH and MCHC significantly increased on week 4 with *Linermis* soluble fractions administrated groups (Table 1).

**Figure 1.** The Antimicrobial activity of *Linermis* different soluble fractions against fungal pathogens. Data are expressed as mean ± SD; n=5. *Significantly different from controls (p<0.05)

The Hb and Ht level did not significantly change with any diet on first week; it significantly increased by the 4th week when fed with 60 mg/kg *L. inermis* injected fishes that are challenged with *A. invadans*. These facts support the present finding that the significant decrease in RBC and Hb content is possibly due to hypochromic microcytic anemia caused by the parasites. The RBC abnormalities such as, viral inclusions, haemoglobin cysts, and haemoparasites are linked to nutritional status [44]. The decreased RBC counts, Ht, and Hb concentrations indicate that RBCs are being
destroyed by the leucocytosis activity leading to erythrocytic anemia [45]. However, A. invadans infected fish fed with control dose without L. inermis had decreased RBC, WBC, and Hb which indicated that RBCs are being destroyed by the leucocytosis with subsequent erythroblastosis [46]. For instance, the pearl spot Etroplus suratensis infected with EUS becomes anemic and subsequently suffered a significant reduction in RBC, Hb, and PCV levels [47]. Similarly Allium sativum influenced erythrocyte, leucocyte, and Ht content in Piaractus mesopotamicus against Anacanthorus penilabiatus [44].

Figure 2. The Total Protein content in C.striatus administered with different doses of L.inermis soluble fractions against A.invadans. Data are expressed as mean ± SD; n=5. *Significantly different from controls (p<0.05)

The MCV, MCH, and MCHC significantly decreased in this study in fish injected with L. inermis. An increase in MCV may be attributed to the swelling of the erythrocytes resulting in amacrocytic anemia or impaired water balance (osmotic stress) or macrocytic anemia in fishes exposed to stress [48]. Similar results were reported in mirgal, brown trout, and rainbow trout fed with supplementation diet against pathogens [49]. The results are in agreement with L. rohita and Oncorhynchus mykiss after dietary administration with A. aspera or garlic against A. hydrophila infection [50].

The serum lysozyme activity was significantly enhanced by the administration of L.inermis mg kg⁻¹ of methanolic soluble fraction on weeks 1 to 4. Significant enhancement was found to be on week 2 to 4 (p <0.05) for 60 mg kg⁻¹ treated groups compared with 0, 6 or 600 mg kg⁻¹ of methanolic sol-

-uble fractions. However, the serum lysozyme activity significantly enhanced A. invadans affected C. striatus with L. inermis soluble fractions from weeks 2 to 4 compared to 0 mg/kg⁻¹ (Control) administration fish (Figure 3).

Lysozyme is a fish defence element, which causes lysis of bacteria and activation of the complement system and phagocytes by acting as opsonin [51]. In the present study, it was observed that the lysozyme activity was well enhanced on treatment with methanolic and water soluble fractions of L. inermis. Similar results of elevated lysozyme activity was observed on 20, 25 and 30 days after feeding Jian carp [52] and large yellow croaker, Pseudoseiwa crocea [53] with Traditional Chinese medicine (TCM) formulated from Astragalus root (Radix astragalin seu heydsari) and Chinese Angelica root (Radix angelicae sinensis) at a ratio 5:1 (w/w). Various authors have reported similar observations of increased values of fish serum lysozyme after activation of the immune system with immune-modulants [54]. The lysozyme activity was reported to have been enhanced on treatment with water or hexane soluble fractions of S. trilobatum leaves in O. mossambicus [55], A. aspera seed in Labeo rohita [56], a marine algae, Dunaliella salina in Oncorhynchus mykiss [57] and four different Chinese herbs (Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica) in Carassius auratus [58] enhanced the lysozyme activity.

This is consistent with most outbreaks of EUS, which tend to be associated with low and declining water
temperatures and high rainfall [59]. This involved identifying mycotic granulomas in histological sections, with further isolation of *A. invadans* from internal tissues in a subset of cases (Level II diagnosis [60]. Snakeheads have been repeatedly described as one of the most EUS susceptible species [61]. Clearly *A. invadans* is infectious to several species of estuarine fish from the mid-Atlantic coast of the USA when inoculated as secondary zoospores [62]. Resistance to infection by *A. invadans* has been reported to occur in tilapia, stickleback and roach [63]. Common carp, *Cyprinus carpio* L., inoculated with *A. piscicida* showed no gross signs of inflammation and mycotic lesions occurred only around the injection site [64]. One type is characterized by the emergence of multinucleate giant cells that engulf hyphae at the site of granulation tissues. In the other type of response, the multi-nucleate giant cell does not engulf the hyphae.

A number of wound healing techniques viz., spraying chemicals into ponds and adding antibiotics in the feed had been used Rehulka [67]. Similar work was carried out using neem paste (*Azaridacta indica*) and aloe paste (*Aloe Vera*) in *Channa striatus* and *Channa punctatus*. Neem paste treated individuals showed complete wound healing on the 6th day of the treatment and the aloe paste treated murrels showed slower recovery in the 8th day of treatment by Haniffa et al., [68].

The serum total protein levels were significantly increased in *A. invadans* infected fish intraperitoneally administrated with 60 mg/kg-1 methanolic soluble fractions of *L. inermis* from weeks 1 to 4 (Figure 2) when compared to the 0 mg/kg-1 (Control) administrated groups, all the statistical values significantly at *p* <0.05. The hemolytic activity of, which has been shown to be increased by *L. inermis* extract in the present study, is known to be one of the powerful non-specific defence mechanisms to protect fish from a wide range of potentially invading organisms such as bacteria, fungi, viruses or parasites [69]. Large yellow croaker fed with TCM, a formulation from Astragalus root and Chinese Angelica root at 1.0% and 1.5% rates, exhibited elevated complement haemolytic activity [52]. The ACH50 of fish that were fed with diets containing marine macro algal-derived k-carrageenan was significantly higher than that of fish fed with a control diet [70].

### 4. CONCLUSION

In conclusion, the present study confirmed that *L. inermis* extract at 60mg/kg-1 dose act as immunostimulant activity and possess positive effect of *A. invadans* disease resistance via enhancing the immune response, including Antimicrobial activities, Hematology, Lysozyme activity, Biochemical analysis and Hemolytic Activity in *C. striatus* against *A.invadans* infection.

### Acknowledgement

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### Conflict of Interest

The authors declare that they have no conflicts of interest.
Table 1. Changes in hematological parameters of C. striatus (mean±SD, n=5) administered with 0, 0.6, 6.0, 60 and 600 mg/kg⁻¹ Linermis soluble fractions against A. invadans.

<table>
<thead>
<tr>
<th>Hematological Parameters</th>
<th>Doses</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10⁵ mm⁻³)</td>
<td>0</td>
<td>2.41±0.34b</td>
<td>2.46±0.35b</td>
<td>2.67±0.38ab</td>
<td>3.11±0.33a</td>
</tr>
<tr>
<td></td>
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<td>2.74±0.37b</td>
<td>3.28±0.35a</td>
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<tr>
<td></td>
<td>6.0</td>
<td>2.95±0.04a</td>
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<td>2.90±0.47a</td>
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<tr>
<td></td>
<td>60</td>
<td>3.43±0.47b</td>
<td>3.79±0.33bc</td>
<td>4.19±0.25b</td>
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<td>600</td>
<td>3.02±0.23a</td>
<td>3.18±0.68a</td>
<td>3.47±0.69a</td>
<td>3.68±0.66a</td>
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<td>WBC (10⁴mm⁻³)</td>
<td>0</td>
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<td>144.3±3.16bc</td>
<td>147.2±2.95b</td>
<td>153.3±1.34a</td>
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<td>144.8±1.92c</td>
<td>150.1±1.20b</td>
<td>150.0±4.52b</td>
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<td></td>
<td>6.0</td>
<td>146.6±2.30c</td>
<td>150.8±3.34b</td>
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<td></td>
<td>60</td>
<td>148.8±0.83c</td>
<td>156.4±2.07b</td>
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<tr>
<td>Haematocrit (%)</td>
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<td>21.38±1.35b</td>
<td>21.41±1.37b</td>
<td>24.28±2.98b</td>
<td>29.28±3.63a</td>
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<td>22.39±1.26b</td>
<td>28.07±3.32a</td>
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<td>5.36±0.25c</td>
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<td>6.21±0.16b</td>
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<td>69.6±1.34ab</td>
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<td>73.4±0.74a</td>
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<tr>
<td>MCH (pg)</td>
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<td>17.4±2.30a</td>
<td>16.0±1.58a</td>
<td>16.2±1.30a</td>
<td>16.4±0.89a</td>
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<tr>
<td></td>
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<td>19.4±1.51a</td>
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</table>

Mean ± SD (n=5) Mean values within the same row sharing the same superscript are not significant different (P>0.05).
References


immunostimulatory effect induced by Lawsonia inermis against Aphanomyces invadans


34. Drabkin DL (1946). Spectrometric studies, XIV-The crystallographic and optimal properties of the hemoglobin of man in comparison with those of other species. Journal of Biological Chemistry; 164: 703-723.


