

## **Acute Toxicity of Aqueous Root Extract of *Withania somnifera* (Ashwagandha) and Its Effects on Biochemical Parameters in *Labeo rohita* Fingerlings**

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### **Abstract**

The present study investigated the acute toxicity of aqueous root extract of *Withania somnifera* (Ashwagandha) in *Labeo rohita* fingerlings. Fish were exposed to concentrations ranging from 200 to 2000 mg/L under static renewal conditions for 96 hours. The 96-h LC<sub>50</sub> value was estimated at 1148.15 ppm through probit analysis ( $R^2 = 0.96$ ), indicating low acute toxicity. Mortality occurred in a dose- and time-dependent manner with a gradual probit slope, suggesting minimal risk of sudden physiological failure. Acute exposure caused significant biochemical changes, including 19% and 24% reductions in carbohydrate and total protein levels, respectively, along with approximately 101.5% and 86.8% increases in AST and ALP activities compared to the control. These alterations reflect a typical stress response at concentrations well above practical dietary inclusion levels. To the best of our knowledge, this appears to be the first report on the LC<sub>50</sub> of *W. somnifera* aqueous extract in fish. The results confirm a wide safety margin and support the safe use of *W. somnifera* as a promising natural feed additive for growth promotion and immunostimulation in *L. rohita* aquaculture.

**Keywords:** *Withania somnifera*, *Labeo rohita*, acute toxicity, LC<sub>50</sub>, biochemical biomarkers, medicinal plants, aquaculture

### **1. Introduction:**

Aquaculture is the fastest-growing food production sector globally, with Indian major carps, particularly *Labeo rohita*, contributing substantially to freshwater fish production in South Asia. The rapid intensification of carp farming has increased the incidence of disease outbreaks and environmental stress, leading to heavy reliance on antibiotics and synthetic chemicals for disease

control and growth promotion (Afroze et al., 2025; Mohammed et al., 2025). However, indiscriminate use of antibiotics has accelerated the emergence of antimicrobial resistance, environmental contamination, and potential risks to human health through residue accumulation in fish tissues (Caputo et al., 2023; Kawsar et al., 2026).

In response to these challenges, medicinal plants have emerged as promising, eco-friendly alternatives to synthetic additives in aquaculture (Dadras et al., 2023; Mbokane & Moyo, 2022). *Withania somnifera* (Ashwagandha), a valuable herb in traditional Ayurvedic medicine, is recognized for its potent antioxidant, immunomodulatory, adaptogenic, and anti-stress properties, primarily attributed to bioactive compounds such as withanolides. Several studies have demonstrated that dietary supplementation of *W. somnifera* root powder or extract at low levels (0.1-2%) significantly improves growth performance, hematological and biochemical profiles, immune responses, and disease resistance against bacterial pathogens such as *Aeromonas hydrophila* in *L. rohita* and other fish species (Debnath & Sahoo, 2025; Laltlanmawia et al., 2019; Sharma et al., 2010; Srivastava et al., 2020).

Despite these encouraging benefits, critical information on the safety profile of *W. somnifera*, particularly the acute toxicity ( $LC_{50}$ ) of its aqueous extract in fish, remains unavailable. To the best of our knowledge, no  $LC_{50}$  data for *W. somnifera* aqueous extract in any fish species have been reported prior to this study.

The present investigation was therefore undertaken to evaluate the acute toxicity of the aqueous root extract of *Withania somnifera* on *Labeo rohita* fingerlings by determining the 96-h  $LC_{50}$  value, recording behavioral and mortality responses, and assessing sub-lethal effects on key biochemical parameters. This study provides the first report on the acute toxicity of *W. somnifera* in fish and aims to establish a scientific safety baseline for its potential use as a dietary supplement in sustainable *L. rohita* aquaculture.

## **2. Materials and Methods:**

**Preparation of aqueous *W. somnifera* extract:** Fresh roots of *Withania somnifera* were procured from a local market in Chh. Sambhajinagar, Maharashtra, India. The roots were washed thoroughly, shade-dried, and ground into a fine powder. Ten grams of powder were homogenized in 50 mL of distilled water using a domestic blender (Fridman et al., 2014). The mixture was then

incubated in a water bath at 60°C for 6 hours with occasional stirring. After cooling, the volume was made up to 100 mL with distilled water. The extract was filtered through muslin cloth followed by Whatman No. 1 filter paper and stored at 4°C in a sterile amber bottle until use. This stock solution was considered as 200,000 mg/L (100%).

**Fish:** Healthy juvenile *Labeo rohita* (standard length:  $3.98 \pm 0.03$  cm; weight:  $2.07 \pm 0.05$  g) were procured from Dadashri Fish Farm, Solapur, Maharashtra, India. Fish were given a prophylactic dip in 0.01% KMnO<sub>4</sub> solution for 15 minutes to prevent dermal infections (Dwivedi & Trivedi, 2015; Magondu et al., 2011). They were then acclimatized for two weeks in rectangular cement tanks (3000 L capacity) containing aerated, chlorine-free well water under natural photoperiod. During acclimatization, fish were fed a commercial pellet feed twice daily. Water quality parameters were monitored regularly. Only healthy and active fish were used for the experiments.

**Acute toxicity test:** The 96-h acute toxicity test was conducted following standard semi-static renewal bioassay procedures (OECD, 1992; APHA, 2017) (Abel & Papoutsoglou, 1986; Audu et al., 2015; Zhao et al., 2014). Ten concentrations (200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, and 2000 mg/L) of the aqueous extract were tested along with a control group (0 mg/L). Each treatment was carried out with 10 fish per concentration. The test was performed in glass aquaria (45.7 × 23 × 45.7 cm) containing 30 L of test solution. Fish were starved for 24 h before and during the exposure period. Test solutions were renewed daily with freshly prepared extract to maintain the desired concentration. Dead fish were removed immediately and mortality was recorded at 24, 48, 72, and 96 h. Water quality parameters (temperature, pH, dissolved oxygen, and alkalinity) were monitored daily. The median lethal concentration (LC<sub>50</sub>) was calculated using probit regression analysis with 95% confidence limits.

**Sub-lethal Exposure and Biochemical Analysis:** Based on the acute toxicity results, fish were exposed to a sub-lethal concentration (approximately 1/3rd of LC<sub>50</sub>) for biochemical studies. At the end of the exposure period, blood samples were collected from the caudal vein using heparinized syringes. Plasma was separated by centrifugation at 10,000 rpm for 20 min at 4°C and stored at 4°C until analysis.

**Estimation of Total Carbohydrates:** Estimated by the anthrone method (Roe, 1955) and expressed as mg/100 mL plasma.

**Estimation of Total Protein:** Determined by the Lowry method (Lowry, 1951) and expressed as µg/mL plasma.

**Estimation of Alkaline Phosphatase:** Measured using a diagnostic kit following the method of Reitman and Frankel (1957) as described by (Chanda et al., 2024).

**Assay of serum aspartate aminotransferase (AST):** Activity was assayed according to (Chanda et al., 2024).

### 3. Results

**Water quality analysis:** During the toxicity tests, the recorded water parameters were (Temperature:  $27.3 \pm 0.4^{\circ}\text{C}$ , pH:  $7.2 \pm 0.2$ , alkalinity:  $264 \pm 0.34$  mg/L, dissolved oxygen:  $7.5 \pm 0.5$  mg/L) were maintained within recommended ranges (Table 1) throughout the experiment.

**Table 1: Physico-chemical analysis of water parameters.**

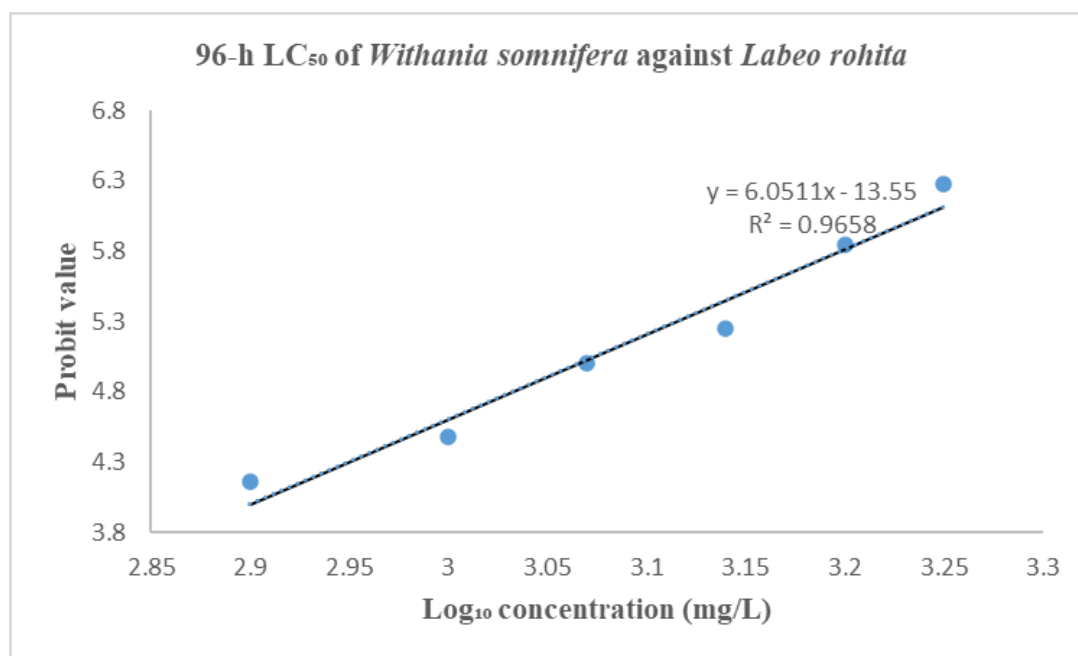
| Parameters       | Mean $\pm$ SD Results | Recommended range |
|------------------|-----------------------|-------------------|
| Temperature      | $27.3 \pm 0.4$        | 18- 35°C          |
| pH               | $7.2 \pm 0.2$         | 6.5- 8.7          |
| Alkalinity       | $264 \pm 0.34$        | 50- 400 mg/L      |
| Dissolved Oxygen | $7.5 \pm 0.5$         | 5-8 mg/L.         |

**Median LC<sub>50</sub> determination:** The number of fish deaths recorded at various concentrations of the extract and at different time intervals are shown in Table 2. The relationship between the aqueous extract concentration and the percentage mortality rate of *L. rohita* at the end of the 96 hrs exposure period has also been indicated (Table 3). No mortality was recorded in the control group.

The 96-h LC<sub>50</sub> of *W. somnifera* was estimated at 1148.15 ppm, indicating low toxicity. The gradual slope of the probit curve suggests that the extract does not induce abrupt physiological failure, reinforcing its safety for prolonged dietary inclusion.

**Table 2:** Number of fish deaths recorded at various concentrations of the extract and at different time intervals

| Sr. No. | Conc (mg/L) or (ppm) | Total No of Rohu fishes | Day-1 24 Hrs          |                          | Day-2 48 Hrs          |                          | Day- 3 72 Hrs         |                          | Day-4 96 Hrs          |                          |
|---------|----------------------|-------------------------|-----------------------|--------------------------|-----------------------|--------------------------|-----------------------|--------------------------|-----------------------|--------------------------|
|         |                      |                         | No of Rohu fish alive | Percentage (%) Mortality | No of Rohu fish alive | Percentage (%) Mortality | No of Rohu fish alive | Percentage (%) Mortality | No of Rohu fish alive | Percentage (%) Mortality |
| 1       | 200                  | 10                      | 10                    | 0                        | 10                    | 0                        | 10                    | 0                        | 10                    | 0                        |
| 2       | 400                  | 10                      | 10                    | 0                        | 10                    | 0                        | 10                    | 0                        | 10                    | 0                        |
| 3       | 600                  | 10                      | 10                    | 0                        | 10                    | 0                        | 9                     | 10                       | 9                     | 10                       |
| 4       | 800                  | 10                      | 10                    | 0                        | 9                     | 10                       | 9                     | 10                       | 8                     | 20                       |
| 5       | 1000                 | 10                      | 10                    | 0                        | 8                     | 20                       | 8                     | 20                       | 7                     | 30                       |
| 6       | 1200                 | 10                      | 9                     | 10                       | 7                     | 30                       | 6                     | 40                       | 5                     | 50                       |
| 7       | 1400                 | 10                      | 9                     | 10                       | 6                     | 40                       | 5                     | 50                       | 4                     | 60                       |
| 8       | 1600                 | 10                      | 9                     | 10                       | 5                     | 50                       | 3                     | 70                       | 2                     | 80                       |
| 9       | 1800                 | 10                      | 8                     | 20                       | 3                     | 70                       | 2                     | 80                       | 1                     | 90                       |
| 10      | 2000                 | 10                      | 8                     | 20                       | 2                     | 80                       | 1                     | 90                       | 0                     | 100                      |



**Fig.1:** The 96 h LC<sub>50</sub> value of *Withania somnifera* for *Labeo rohita* was estimated to be approximately 1148.15 ppm based on probit regression analysis ( $R^2 = 0.96$ ).

**Table 3:** Relationship between plant aqueous extract concentration and the percentage mortality of *L. rohita* at the 96 hrs exposure period and LC<sub>50</sub> at 96 Hrs.

| Sr. No | Conc (mg/L) | log 10 conc | Total No of Rohu Fishes | No of Rohu fish alive | Percentage (%) Mortality | Probit value |
|--------|-------------|-------------|-------------------------|-----------------------|--------------------------|--------------|
| 1      | 200         | 2.30        | 10                      | 10                    |                          | 3.04         |
| 2      | 400         | 2.60        | 10                      | 10                    | 0                        | 3.04         |
| 3      | 600         | 2.77        | 10                      | 9                     | 10                       | 3.72         |
| 4      | 800         | 2.90        | 10                      | 8                     | 20                       | 4.16         |
| 5      | 1000        | 3           | 10                      | 7                     | 30                       | 4.48         |
| 6      | 1200        | 3.07        | 10                      | 5                     | 50                       | 5            |
| 7      | 1400        | 3.14        | 10                      | 4                     | 60                       | 5.25         |
| 8      | 1600        | 3.20        | 10                      | 2                     | 80                       | 5.84         |
| 9      | 1800        | 3.25        | 10                      | 1                     | 90                       | 6.28         |
| 10     | 2000        | 3.30        | 10                      | 0                     | 100                      | 6.96         |

#### Biochemical Analysis (Biomarker):

The biochemical parameters significant alterations in biochemical biomarkers were observed between the control and experimental groups. The carbohydrate content decreased 19% from  $98.23 \pm 0.28$  mg/100 ml in the control group to  $79.68 \pm 0.12$  mg/100 ml in the experimental group. Similarly, total protein level declined 24% from  $2.51 \pm 0.51$  µg/ml in the control to  $1.91 \pm 0.03$  µg/ml in the experimental group. In contrast, the activity of AST increased 101.5 % from  $28.43 \pm 0.53$  IU/L in the control group to  $57.31 \pm 0.07$  IU/L in the experimental group.

**Table 4:** Biochemical parameters in control and experimental group

| Biochemical Parameters      | Control          | Experiment       |
|-----------------------------|------------------|------------------|
| Carbohydrate (mg/100 ml)    | $98.23 \pm 0.28$ | $79.68 \pm 0.12$ |
| Total Protein (µg/ml)       | $2.51 \pm 0.51$  | $1.91 \pm 0.03$  |
| AST (IU/L)                  | $28.43 \pm 0.53$ | $57.31 \pm 0.07$ |
| Alkaline Phosphatase (IU/L) | $18.36 \pm 0.27$ | $34.3 \pm 0.41$  |

Likewise, alkaline phosphatase activity was elevated 86.8 % from  $18.36 \pm 0.27$  IU/L in the control group to  $34.3 \pm 0.41$  IU/L in the experimental group. These results indicate significant biochemical alterations in the experimental fish compared to the control group.

#### 4. Discussion:

The physico-chemical parameters of aquarium water, including temperature ( $27.3 \pm 0.4^{\circ}\text{C}$ ), pH (7.2-7.7), alkalinity (264-284 mg/L), and dissolved oxygen (6.5-7.5 mg/L), remained within the recommended ranges for *Labeo rohita* throughout the experimental period. This stability confirmed that the observed effects were due to the aqueous extract of *Withania somnifera* rather than environmental stressors.

The acute toxicity test revealed a 96-h  $\text{LC}_{50}$  of approximately 1148.15 ppm for the *W. somnifera* aqueous extract in *L. rohita*. This relatively high  $\text{LC}_{50}$  value classifies the extract as having low acute toxicity. No mortality was recorded in the control group, while mortality increased in a dose- and time-dependent manner at higher concentrations ( $\geq 600$  mg/L). The gradual slope of the probit regression curve ( $R^2 = 0.96$ ) indicates that the extract does not cause sudden or catastrophic physiological disruption, even at elevated concentrations. This supports its potential safety for use in aquaculture, as concentrations required for beneficial dietary supplementation are typically far below this threshold.

The aqueous extract of *Withania somnifera* exhibited low acute toxicity in *Labeo rohita*, with a 96-h  $\text{LC}_{50}$  of 1148.15 ppm. Available data across other fish species also indicate a consistently safe profile. In Nile tilapia (*Oreochromis niloticus*), dietary supplementation of *W. somnifera* aqueous extract (up to 3 mL/kg body weight) was safely used to alleviate cadmium-induced toxicity without reported adverse effects (El-Sabbagh et al., 2022). In African catfish (*Clarias batrachus*), aqueous root extract at 100 mg/kg body weight effectively ameliorated organochlorine-induced renal toxicity (Chand & Singh, n.d.; Tchounwou et al., 2012). Similar tolerance has been observed in common carp (*Cyprinus carpio*) and *Catla catla* at dietary inclusion levels of 0.1-2% (Habib et al., 2024; Thayes et al., 2024). These findings, combined with the high  $\text{LC}_{50}$  and gradual probit slope in *L. rohita*, demonstrate the wide safety margin of *W. somnifera* across multiple cultured fish species.

Previous studies on *W. somnifera* (Ashwagandha) in aquaculture have consistently demonstrated its beneficial effects as an immunostimulant, growth promoter, and stress mitigator in *L. rohita* and other species when incorporated at low dietary levels, with enhanced disease resistance against pathogens such as *Aeromonas hydrophila* (Sharma et al., 2010, 2017; Srivastava et al., 2020).

Biochemical analysis revealed significant alterations, carbohydrate content decreased by ~19% and total protein levels declined by ~24%. These reductions suggest enhanced catabolic activity or energy mobilization in response to stress, as fish often utilize stored glycogen and proteins to meet increased metabolic demands during xenobiotic exposure (Oner et al., 2008).

Conversely, aspartate aminotransferase (AST) activity increased markedly (~101.5% , to  $57.31 \pm 0.07$  IU/L), and alkaline phosphatase (ALP) rose by ~86.8% (to  $34.3 \pm 0.41$  IU/L). Elevated transaminase (AST) and phosphatase (ALP) activities are established biomarkers of hepatic or tissue stress and mild cellular damage in fish exposed to various stressors or plant extracts. These elevations likely reflect compensatory responses or minor leakage from hepatocytes due to altered membrane permeability. Similar patterns decreased plasma proteins/carbohydrates alongside increased enzyme activities have been reported in fish under different toxicant exposures, indicating a generalized stress response (Laltlanmawia et al., 2019). Importantly, the observed biochemical shifts occurred under toxicity test conditions (near or at sub-lethal levels), which are intentionally much higher than those used in dietary supplementation studies. In practical aquaculture applications, dietary inclusion of *W. somnifera* at optimized low levels has been shown to improve serum protein profiles, hematological parameters, immune responses, and overall health without inducing such stress markers (Laltlanmawia et al., 2019; Sharma et al., 2010, 2017).

The high  $LC_{50}$  value, gradual dose-response relationship, and consistent safety across species reinforce that *Withania somnifera* aqueous extract possesses a wide safety margin. This makes it suitable for prolonged dietary inclusion as a natural growth promoter and immunostimulant in *L. rohita* and other cultured fish species. Further chronic exposure and feeding trials at nutritionally relevant doses are recommended to fully confirm the absence of cumulative effects and support its integration into sustainable aquaculture practices.

## 5. Conclusion

The present study provides the first report on the acute toxicity of *Withania somnifera* aqueous extract in any fish species, with a 96-h  $LC_{50}$  value of 1148.15 ppm in *Labeo rohita*. The extract exhibited low acute toxicity and a gradual dose-response relationship. Although acute exposure caused some biochemical alterations indicative of stress, these changes occurred at concentrations

well above practical dietary levels. Compared to other botanicals such as Neem and Garlic, *W. somnifera* showed a markedly wider safety margin. These results support the safe use of *Withania somnifera* as a promising natural feed additive for growth promotion and immune-stimulation in *L. rohita* aquaculture at optimized low dietary inclusion levels. Further long-term feeding trials are recommended to validate its sustained benefits.

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