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Short communication

# Effect of *Cucurbita mixta* (L.) seed meal enrichment diet on growth, immune response and disease resistance in *Oreochromis mossambicus*

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ABSTRACT

The impact of *Cucurbita mixta* (L.) seed meal enriched diet on growth performance, innate immune response, and disease resistance in *Oreochromis mossambicus* against *Aeromonas hydrophila* was investigated. *O. mossambicus* was fed with 2 g $kg^{-1}$, 4 g $kg^{-1}$, and 6 g $kg^{-1}$ *C. mixta* seed meal diets for a period of 4 weeks. The results indicated that *C. mixta* seed meal diets at 4 g $kg^{-1}$ and 6 g $kg^{-1}$ significantly ($P < 0.05$) enhances the survival rate, weight gain (WG), protein efficiency ratio (PER), specific growth rate (SGR), feed conversion ratio (FCR), and feed efficiency (FE) from weeks 1–4 when compared to control. *C. mixta* seed meals administered as feed supplements significantly ($P < 0.05$) enhanced the complement activity, phagocytic activity, respiratory burst activity, and lysosome activity in infected fish fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* seed meal enriched diet from weeks 2–4. The cumulative mortality was lower in the fish fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* seed meal enriched diets (15% and 18%) than with 2 g $kg^{-1}$ diet (26%). The present investigation suggested that *C. mixta* seed meal enriched diet at 4 g $kg^{-1}$ and 6 g $kg^{-1}$ enhance the better growth performance, innate immunity, and disease resistance against *A. hydrophilla* in *O. mossambicus*.



## 1. Introduction

*Aeromonas hydrophila* is a heterotrophic, free-living, motile, Gram-negative bacterium, commonly found in freshwater and occasionally in marine waters [1]. This organism may also be found in areas where the climate is warm, in saltwater, estuarine, chlorinated and unchlorinated waters, and aerobic and anaerobic environments [2–4]. *A. hydrophila* comprises part of the normal microbial flora of freshwater fish, but is an opportunist pathogen, being converted from a commensal to a pathogenic state under stress conditions [5]. Traditionally, *A. hydrophila* has been recognized as the causative agent of haemorrhagic septicaemia/motile aeromonas septicaemia, skin ulceration, fin/tail rot and red sore disease in fishes [6–8]. The pathogen has the ability to adhere to selected host T-cells via the action of 'adhesions' [4,9]. These adhesions appear extremely selective, recognizing D mannose and L fructose side chains on the surface of eukaryotic cells. With attachment, the host becomes at the mercy of the pathogen [3,4].

Presently, medicinal plants are widely used as chemotherapeutics and feed additives [10]. They have the properties of growth promoting ability, a tonic to improve the immune system, antimicrobial capability, and stimulating appetite and anti-stress characteristics [11]. Several plants or their byproducts contain phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine, and polypeptide compounds, many of which are effective alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds [12]. In addition, medicinal plants are rich in a wide variety of nutrients [10] used for feed additives [11,12]. *Cucurbita mixta* (pumpkin seeds) have long been used as sources of nutrition. Based on its nutrition content, it is possible that the seeds of this plant used as immunonutrient. Pumpkin seeds are rich in essential fatty acids such as

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palmitic, oleic, linoleic and steric acids as well as arginine and glutamic acid. It was reported that the seed contains in rich essential fatty acids on anti-obesity effects and helps to prevent the formation of renal calculi and chronic severe wound infection in diabetic condition [13].

The incorporation of whole plant or parts (leaf, root or seed) or extract compounds with animal feeds significantly enhances the growth performance, innate immune response, and disease resistance in several finfishes and shellfishes [14–17]. According to Van Hai [18], medicinal plants have been proven as growth promoters. Firstly, they enhance digestive enzymes, and thus boost survival and growth rates of aquatic animals. Three herbs (*Alteranthera sessilis*, *Eclipta alba*, and *Cissus quadrangularis*) acted as appetizers and enhanced the activities of digestive enzymes (protease, amylase, and lipase) of freshwater prawn [16]. This resulted in an enhancement of food utilization and ultimately led to better growth rates as indicated by the evidence of elevated concentrations of vitamins, protein, essential amino acids, unsaturated fatty acids, and minerals. Vitamin level of C and E and sodium and potassium increased when the prawns were fed diets supplemented with herbs.

Traditional Chinese medicines had a beneficial effect on the growth of common carp [19]. The herbal mixture of *Massa medicata fermentata*, *Crataegi fructus*, *Artemisia capillaries*, and *Cnidium officinale* enhanced the growth and fatty acid utilization of Japanese flounder [20]. Diets supplemented with *Ocimum sanctum* and *Withania somnifera* improved the growth and FCR of juvenile greasy groupers [21]. The dietary ginseng herb (Ginsana® G115) enhanced the growth performance and food utilization efficiency of Nile tilapia [22]. Five herbal extracts (*Cynodon dactylon*, *Piper longum*, *Phyllanthus niruri*, *Tridax procumbens*, and *Zingiber officinalis*) increased the survival and growth rate of *Epinephalus tauvina* [23].

To the best of our knowledge, this is the first study focusing on the effects of *C. mixta* seed meal enrichment diet on growth performance, innate immune function, and disease resistance against bacterial diseases. Therefore, the present experiment evaluates the impact of dietary administration of *C. mixta* seed meal on growth performance, innate immune function, and disease resistance in *O. mossambicus* against *A. hydrophilla*.

## 2. Materials and methods

### 2.1. Diet

The basal diet (control) comprised of mackerel meal, dehulled soybean meal, and corn gluten meal as the protein sources; wheat flour, α-potato starch, and wheat gluten as carbohydrate and fish oil as lipid source in addition with vitamin and mineral premix as described in Table 1. The dietary supplement (*C. mixta*) were incorporated separately (four experimental diets) with the basal diet at doses of 2, 4, and 6 g $kg^{-1}$ by evenly mixing with thoroughly (Table 2). The enriched feeds were dried in a vacuum freeze drier for 15 h, ground, and extruded by passing through 5 mm mesh sieve. The prepared diets were stored at −20 °C until used for the experiment. The proximate composition of the experimental diets quantified following AOAC [24] method comprised the percentage of crude protein, crude lipid, crude carbohydrate, crude ash, and crude fiber.

### 2.2. Pathogen

*A. hydrophila* (MTCC 646) was obtained from Institute of Microbial Technology in Chandigarh, India which was isolated from infected fish. The pathogenic of *A. hydrophila* was confirmed to inoculate into *O. mossambicus* and reisolation according to Krieg and Hold [25,26] *A. hydrophila* was grown with agitation at 37 °C in a 250 ml conical flask containing tryptic soy broth (TSB; Merck) to log phase. The culture was harvested by centrifugation at 3500 × g for 20 min at 4 °C. Bacterial pellets were washed twice with sterile 0.15 M phosphate buffered saline (PBS) at pH 7.2. The bacterial pellets were resuspended and divided into aliquots and stored in (TSB) supplemented with 15% (v/v) glycerol at −70 °C until used. The identity of the bacterium was confirmed by morphological, pictorial, and biochemical characteristics [27] and the followed by PCR for confirmation of genus and species, using the methods described by Ghatak et al. [28].

**Table 1**
Ingredients and proximate composition (g) of experimental diets.

| Ingredients (g) | Diets groups (%) | | | | |
|---|---|---|---|---|---|
| | C | I | 2 g $Kg^{-1}$ | 4 g $Kg^{-1}$ | 6 g $Kg^{-1}$ |
| Mackerel meal | 55 | 55 | 55 | 55 | 55 |
| Dehulled soybean meal | 12 | 12 | 12 | 12 | 12 |
| Corn gluten meal | 5 | 5 | 5 | 5 | 5 |
| Wheat flour | 12 | 12 | 12 | 12 | 12 |
| α-potato starch | 2 | 2 | 2 | 2 | 2 |
| Wheat gluten | 6 | 6 | 4 | 2 | 0 |
| Fish oil | 5 | 5 | 5 | 5 | 5 |
| Vitamin premix (mg)[a] | 2 | 2 | 2 | 2 | 2 |
| Mineral premix (mg)[b] | 1 | 1 | 1 | 1 | 1 |
| *Cucurbita mixta* seed meal | 0 | 0 | 2 | 4 | 6 |

[a] Vitaminpremix per kg: Vitamin A = 700000 IU; Vitamin D = 140000 IU; Vitamin E = 500 mg; Vitamin $B_{12}$ = 1000 mcg; FolicAcid = 100 mg; Nicotinamide = 1000 mg.
[b] Mineralpremix per kg:Copper = 1200 mg; Cobalt = 150 mg; Iron = 1500 mg; Zinc = 3000 mg; Iodine = 325 mg; Selenium = 10 mg; Magnesium = 6000 mg; Manganese = 1500 mg; Potassium = 100 mg; Calcium = 270 gm; Phosphorus = 130 gm; Sulphur = 7.2 gm; Fluorine = 300 mg.

### 2.3. Fish and experimental design

The freshwater fish, *O. mosambicus* (30.5 ± 1.7 g) were procured from a commercial fish farm; the fishes were maintained in 60 L aerated fiber tanks. They were examined for their health status immediately upon arrival. After two week's acclimation, the fishes were divided into five experimental groups of 25 each in triplicate (5 × 25 x 3 = 375 fish) and the fishes were fed with (i) control group, without *C. mixata* seed meal diet (C), (ii) infected group, fed without *C. mixata* seed meal diet (I), (iii) infected, fed with 2 g $kg^{-1}$ of *C. mixata* seed meal supplementation diet, (iv) infected, fed with 4 g $kg^{-1}$ of *C. mixata* seed meal supplementation diet, and (v) infected, fed with 6 g $kg^{-1}$ of *C. mixata* seed meal supplementation diet at the rate of 5% of their body weight twice a day. Feeding with the respective diets continued till the end of experiment. On 30$^{th}$ day of feeding, all groups except control group were challenged intraperitoneally (i.p.) with 100 μl PBS containing *A. hydrophila* at 3.1 × $10^7$ cfu $ml^{-1}$ as determined our previous study using a Neubauer haemocytometer. On weeks 1, 2, and 4 post-infection, six fishes were randomly collected from each experimental tank to collect blood samples for immunological assays after anaesthetizing with MS-222 ($NaHCO_3$ and tricaine methane sulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min.

### 2.4. Growth performance

The growth performance, including percentage weight gain (RGR), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) for each group was determined by Olmedo Sanchez et al. [29].

**Table 2**
Proximate composition of different experimental diets.

| Experimental Groups | Moisture (%) | Crude protein (%) | Crude lipid (%) | Crude fiber (%) | Crude ash (%) |
|---|---|---|---|---|---|
| Control diet | 7.12 | 54.30 | 8.60 | 3.60 | 7.60 |
| Negative Control diet | 7.16 | 53.40 | 8.70 | 3.40 | 7.40 |
| ***Cucurbitta mixta* seed meal enriched diet** | | | | | |
| 2 g $kg^{-1}$ | 8.00 | 53.20 | 6.35 | 5.70 | 9.15 |
| 4 g $kg^{-1}$ | 8.12 | 50.40 | 6.10 | 4.35 | 8.50 |
| 6 g $kg^{-1}$ | 8.10 | 53.00 | 5.70 | 6.45 | 12.40 |

Specific growth rate (SGR) = (Ln Final weight - Ln Initial weight) ÷ No of days in trial × 100

Feed conversion ratio (FCR) = Feed given (dry wt) ÷Weight gain (wet weight)

Protein efficiency ratio (PER) = wet weight gain by fish (g) ÷ Protein intake (g)

$$\text{Cumulative mortality}(\%) = \frac{\text{Total mortality in each treatment after challenge}}{\text{Total number of fish challenged for same treatment}} \times 100$$

$$\text{Relative percent survival}(\text{RPS}) = 1 - \frac{(\%\text{ of Mortality in treated group})}{(\%\text{ of Mortality in control group})} \times 100$$

### 2.5. Preparation of serum and head kidney macrophages

*O. mosambicus* were sacrificed with an overdose of anesthetic and exsanguinated by caudal vein puncture using 1 ml capacity Vacuettes containing a Z Serum Sep Clot Activator (Greiner Bio-one). A part of blood samples were kept separately and were maintained at −70 °C until used for the experiment. Another part of blood samples were allowed to clot for 2 h at 4 °C and the serum was separated by centrifugation at 3500*g* for 25 min at 4 °C. The samples were maintained at −70 °C for subsequent analysis. The head kidney macrophages were isolated and prepared for the evaluation of immunological parameters according to Secombes [30].

### 2.6. Immunological assays

The phagocytic activity of blood was determined following Anderson et al. [31]. Reactive oxygen species (ROS) production of the intracellular respiratory burst activity was measured by NBT method according to Anderson et al. [31]. The alternative complement activity was examined by the following method of Yano [32] using rabbit red blood cells (RBC; Oxoid); the lysozyme activity was determined by turbidimetric assay as described by Anderson et al. [31].

### 2.7. Challenge study with A. hydrophila

After 30 days of feeding trial, 20 fish in each treatment group were maintained separately and challenged with virulent *A. hydrophila* as mentioned above dose. The bacterial culture, challenge study, and the concentration of bacterial suspension were studied as mentioned previously. Mortality was observed for 30 days. The tissues were collected from the dead fish for bacteriological study to confirm *A. hydrophila* as the cause of death. The cumulative and relative percent survival (RPS) in different treatment groups were calculated as follows [33].

### 2.8. Statistical analysis

The data of each parameter were expressed as the mean ± standard error of mean (SEM) and the effects of experimental diets were tested using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test using SPSS (version 16 for windows). Differences were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Growth performance and feed utilization

Growth performance of *O. mossambicus* fed with *C. mixata* seed meal enriched diets were measured at the end of fourth week (Table 3). All the enriched diet fed groups were found active and healthy. The survival rate of *C. mixata* seed meal enriched diet fish groups, ranged between 89.7 and 93.7%. At the end of the feeding trial, the infected fishes were fed with 4 g or 6 g $kg^{-1}$ *C. mixata* seed meal enriched diets and showed better growth rate when compared to control and 2 g $kg^{-1}$ *C. mixata* seed meal supplementation diet. A significant ($P < 0.05$) difference was found in the growth performance across the different concentrations of *C. mixata* seed meal diets. A significant ($P < 0.05$) increase in the feed conversion ratio (FCR), specific growth rate (SGR), feed efficiency (FE), and protein efficiency ratio (PER) was observed in fish fed with 4 g or 6 g $kg^{-1}$ *C. mixata* seed meal diets over the control group (Table 3).

### 3.2. Phagocytic activity

The phagocytic activity in head kidney leucocytes did not

**Table 3**
Mean growth performance and feed utilization after fourth week of *O. mossambicus* fed *C. mixta* seed meal supplementation diets against *A. hydrophila*.

| Parameter | Control | I | 2 g $kg^{-1}$ | 4 g $kg^{-1}$ | 6 g $kg^{-1}$ |
|---|---|---|---|---|---|
| IBW(g) | 31.5 ± 1.3 | 29.8 ± 1.23 | 33.4 ± 1.1* | 33.7 ± 2.4* | 32.0 ± 1.4 |
| FBW(g) | 40.9 ± 1.8 | 32.7 ± 1.85 | 42.3 ± 2.0* | 48.1 ± 2.4* | 49.9 ± 2.0* |
| SGR (% $d^{-1}$) | 2.16 ± 0.02 | 0.80 ± 0.01 | 2.16 ± 0.02 | 2.26 ± 0.03* | 2.94 ± 0.01* |
| FCR | 0.80 ± 0.02 | 0.95 ± 0.02 | 0.90 ± 0.02 | 0.89 ± 0.03* | 0.90 ± 0.03* |
| FE | 1.59 ± 0.02 | 1.06 ± 0.03 | 1.94 ± 0.02* | 1.91 ± 0.03* | 1.82 ± 0.02* |
| PER | 4.00 ± 0.03 | 2.94 ± 0.04 | 3.78 ± 0.02 | 3.83 ± 0.02 | 3.76 ± 0.02 |
| Survival rate (%) | 100 ± 0.0 | 15.5 ± 0.12 | 91.7 ± 0.14 | 91.3 ± 0.12 | 89.7 ± 0.14 |

The values are expressed as mean ± standard errors of mean. Means in a given row with different superscript letters were significantly different at P < 0.05*.
IBW= Initial body weight, FBW = Final body weight, SGR = Specific growth rate, FCR = Feed conversion ratio, FE= Feed efficiency ratio, PER = Protein efficiency ratio.

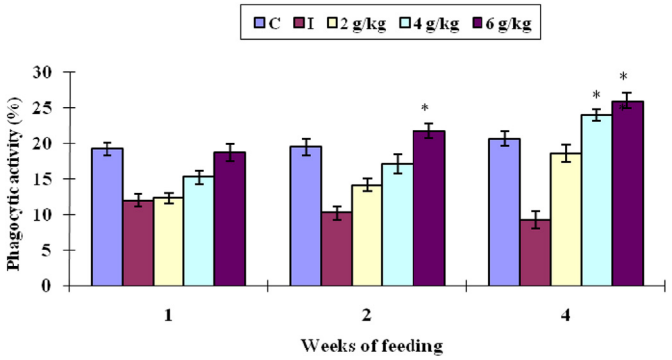


**Fig. 1.** Phagocytic activity (%) of *O. mossambicus* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentrations (2, 4, and 6 g $kg^{-1}$) of *C. mixta* against *A. hydrophila*. Significant difference (P < 0.05) from the control is indicated by asterisks.

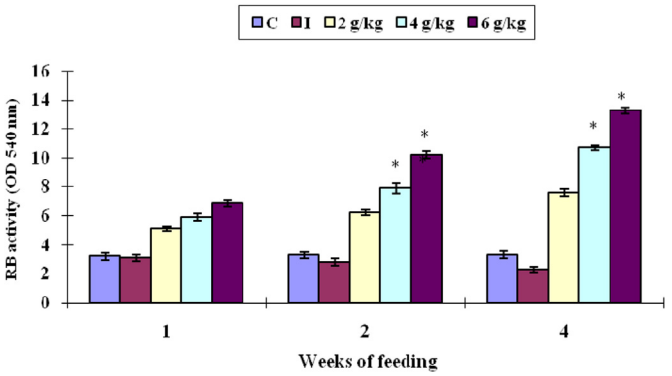


**Fig. 3.** Respiratory burst (RB) activity of *O. mossambicus* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentrations (2, 4, and 6 g $kg^{-1}$) of *Cucurbita mixta* against *A. hydrophila*. Significant difference (P < 0.05) from the control is indicated by asterisks.

significantly vary in any *C. mixata* seed meal supplementation diets fed group on first week. Whereas, the infected fish fed with 4 g and 6 g $kg^{-1}$ *C. mixata* seed meal diets, the phagocytic activity was significantly (P < 0.05) elevated on weeks 2 and 4 when compared to the control but not with 2 g $kg^{-1}$ *C. mixata* seed meal supplementation diet (Fig. 1).

### 3.3. Complement activity

The complement activity was significantly increased (P < 0.05) in the infected fish fed with 4 g and 6 g $kg^{-1}$ *C. mixata* seed meal diets from week 1–4 when compared to control. Whereas, only a moderate increase of complement activity was observed in 2 g $kg^{-1}$ *C. mixata* seed meal diet from week 1–4 (Fig. 2).

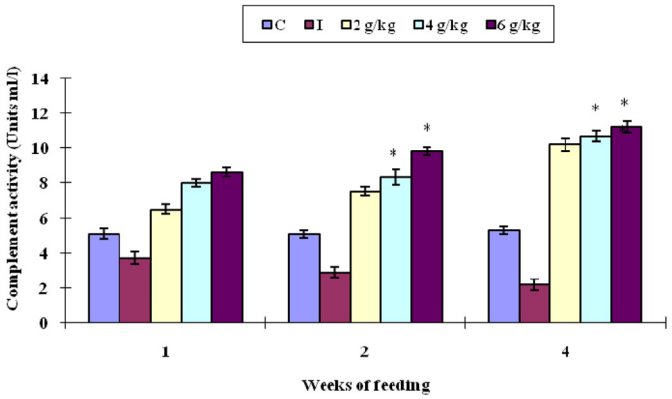


**Fig. 2.** Complement activity of *O. mossambicus* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentrations (2, 4, and 6 g $kg^{-1}$) of *Cucurbita mixta* against *A. hydrophila*. Significant difference (P < 0.05) from the control is indicated by asterisks.

### 3.4. Respiratory burst activity

Maximum respiratory burst activities of phagocytes were observed in 6 g $kg^{-1}$ on week 4 followed by 4 g $kg^{-}$ *C. mixata* enriched diets, respectively. The result revealed that the respiratory burst activity was significantly elevated (P < 0.05) with 4 and 6 g $kg^{-1}$ enriched diets on weeks 2 and 4 but did not on first week (Fig. 3).

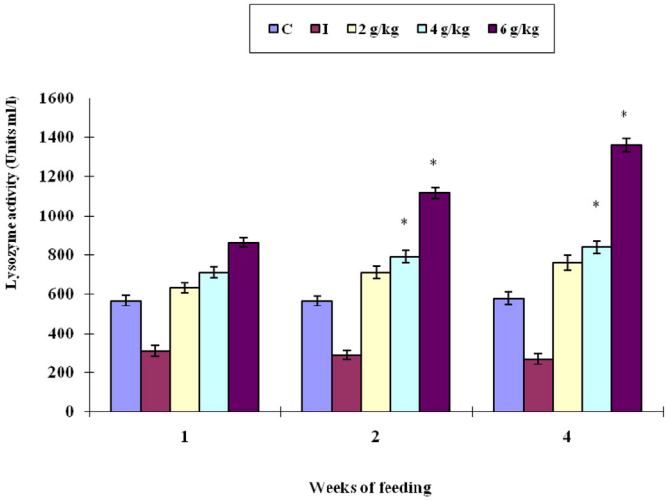


**Fig. 4.** Lysozyme activity of *O. mossambicus* (mean ± SEM, n = 6) fed dietary supplementation diets with different concentrations (2, 4, and 6 g $kg^{-1}$) of *Cucurbita mixta* against *A. hydrophila*. Significant difference (P < 0.05) from the control is indicated by asterisks.

### 3.5. Lysosome activity

The lysosome activity was significantly increased ($P < 0.05$) in 4 and 6 g $kg^{-1}$ *C. mixata* enriched diet groups from weeks 1–4 when compared to control but did not 2 g $kg^{-1}$ *C. mixata* seed meal diet groups (Fig. 4).

### 3.6. Disease resistance

The cumulative mortality was 15% and 18% in infected fish fed with 4 and 6 g $kg^{-1}$*C. mixta* seed meal supplementation diets. The mortality was high (26%) when fed with 2 g $kg^{-1}$ diet. However, the maximum mortality was observed (90%) when infected fish were fed with non *C. mixta* seed meal diet for 30 days (Fig. 5).

## 4. Discussion

In aquaculture, application of traditional medicine is more advantageous to overcome the drawbacks in traditional chemotherapy; hence application of natural mineral supplements or herbals with multi-functional active principles can be ideal alternatives. In the present study a pioneering attempt was made to document the impact of dietary administration of *C. mixta* seed meal to enhance the growth, survival and immuno-modulatory response of freshwater fish, *O. mossambicus* against *A. hydrophila*. As far as our knowledge goes, there were no detailed studies on growth performance and immune response in aquatic species of *C. mixta* seed meal supplementation diets. The optimum feeding regimes/schedules of cultured fish is an important aspect in achieving efficient production and also could lead to significant saving in diet cost. Diet supplementation is an important aspect in aquaculture management especially in intensive or in semi-intensive fish culture, and is promising for increasing fish production. In aquaculture, diet is often the single largest operating cost item and can represent over 50% of the operating costs in intensive aquaculture [34].

In aquaculture, various growth-promoting additives are commonly added to the diets to improve the nutrient utilization, growth performance, and survival of cultured fish. Dietary supplements include probiotics, yeast, amino acids, antioxidants, carnitine, colourants, enzymes, lipid derivatives, nutraceutics, vitamins, hormones, aromatic compounds, plant extracts and certain organic acids/salts [22]. An improvement of the growth parameters, WG, SGR and FCR were observed in catfish fed diet containing, *O. vulgare* essential oil [35]. The oral administration of *A. aspera* [36] increased the SGR value and decreased the FCR value in *L. rohita* and similar results have been reported in *C. carpio* fed *Rheum officinale* enriched diets [37]. Moreover, the survival in *L. rohita* was improved by feeding diets with *C. longa* [38].

In the present study, fish fed with different concentrations of *C. mixta* seed meal show significant difference in growth performance. Only fish group fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ showed the better growth performance when compared to control fish group. Recently, Ojha et al. [39] reported that the *M. pruriens* seed meal significantly enhances growth performance, metabolic activity, and immune response of freshwater fish, *Labeo rohita*. At the same time, Siddhuraj and Becker [40] stated that the higher inclusion rate of mucuna seed meal significantly reduced the growth parameters in the freshwater fish, *Cyprinus carpio* because most of the plant-based feed stuffs have a wide variety of anti-nutritional factors such as phytin, non-starch polysaccharides (NSP), and protease inhibitors, which may impair nutrient utilization, as well as impair fish performance and health [41]. Lakshmi Bai and Kumar Reddy [42] stated that, 30% of cottonseed meal diet showed satisfactory results regarding the growth performance of red bellied Pacu, *Piaractus brachypomus*.

Enhancement of the immune system is the most promising strategy in preventing fish diseases. The nonspecific immune system of fish is considered to be the first line of defense against invading pathogens, and is more important for fish rather than mammals [42]. The nonspecific immune response depends on the function of serum and macrophage activity such as phagocytosis and chemotaxis. Phagocytic cells are the most important cellular components of the innate immune system of fish [35]; their phagocytic activity constitutes a primitive defense mechanism [44] which is an important characteristic of the nonspecific immune system [45]. In the present study, infected *O. mosambicus* fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* supplementation diet groups were able to significantly enhance the phagocytic activity of leukocytes on weeks 2 and 4. However, no significant effect was found with any diet during first week.

The complement system is a major antimicrobial defense system of innate immunity, providing vital host defense, especially early after infection before adaptive immunity is activated. It is crucial for phagocytes recruitment, clearance of invading pathogens, and elimination of altered cells. It serves as a bridge between innate immunity and adaptive immunity [35,40]. In the present study the complement activity was significantly enhanced on fish groups fed 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* supplementation diets.

Respiratory burst (sometimes called oxidative burst) is the rapid

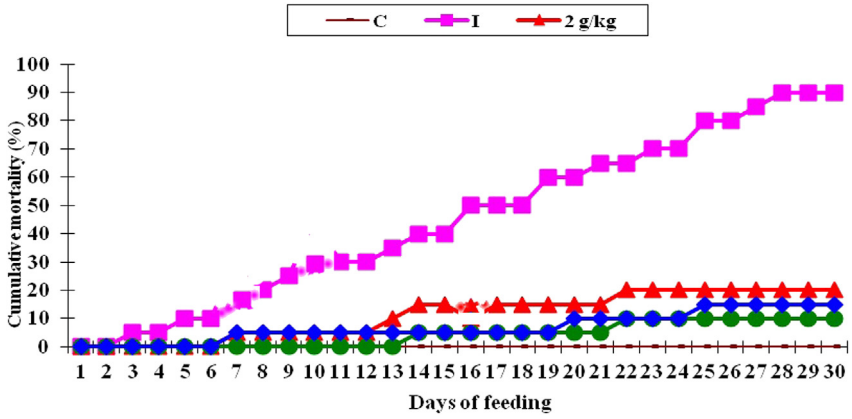


**Fig. 5.** Cumulative mortality (%) of *O. mossambicus* (n = 20) fed dietary supplementation diets for 30 days with difference concentrations (2, 4, and 6 g $kg^{-1}$) of *Cucurbita mixta* against *A. hydrophila*. Significant difference ($P < 0.05$) from the control is indicated by asterisks.

release of reactive oxygen species (superoxide radical and hydrogen peroxide) from different types of cells. Stimulation of the phagocytic cell membrane and thereby activation of the membrane associated NADPH oxidase, initiates an increased oxygen consumption and the production of reactive oxygen species (ROS) which are considered to be toxic for bacterial pathogens [46,47]. In the present study, infected *O. mosambicus* fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* supplementation diet groups were able to significantly enhance the respiratory burst activity.

Lysozyme is a cationic protein that is present in mucus, lymphoid tissue, plasma as well as in other fluids and is also expressed in a wide variety of tissues [48]. In fish, lysozyme is synthesized in both liver and extra hepatic sites [35] and involved in a broad range of defense mechanisms such as bacteriolysis, opsonisation, immune response, antimicrobial as well as restricted antiviral and antineoplastic activity as found in higher vertebrates [40,43,45]. In the present study, infected *O. mosambicus* fed with 4 g $kg^{-1}$ and 6 g $kg^{-1}$ of *C. mixta* supplementation diet groups were able to significantly enhance the lysosome activity. The cumulative mortality was 15% and 18% in infected fish fed with 4 and 6 g $kg^{-1}$ *C. mixta* seed meal supplementation diets, respectively. Present study clearly demonstrated that *C. mixta* seed meal supplementation diets, enhances the immune response against the Gram-negative bacterium, *A. hydrophila*.

To the best of our knowledge, there were no detailed immunological studies in aquatic animal species using supplementation diet with *C. mixta* seed meal. Hence we conclude that *C. mixta* can be used as enriched diets in stimulating immunity for effective production of economically valuable freshwater fish, *O. mossambicus.* Further detailed immunological and molecular studies are needed to strengthen the role of this herbal compounds in aquaculture before recommending the *C. mixta* seed meal supplementation diets as potential immunostimulant in other cultured fish species against pathogens.

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