Effect of dietary *Medicago sativa* extract on hepato-renal indicators, intestinal mucosal morphology and serum bactericidal activity in *Cyprinus carpio* fingerlings

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ABSTRACT

Plant extracts and powders have recently been used as alternatives to chemical drugs in aquaculture, but due to the presence of antinutritional factors, their use is still limited and dose-dependent. To address this issue, the present study intended to investigate the effects of Alfalfa, *Medicago sativa* extract on health status in terms of hepato-renal enzymes, intestinal mucosal health, and serum bactericidal activity in common carp, *Cyprinus carpio* fingerlings. Fish (weighing 6.51 ± 0.3 g) were divided into five dietary treatment groups and fed with experimental diets for 60 days. Following completion of the experimental trial, final weight, hepato-renal indicator enzymes, intestinal mucosal morphology and serum bactericidal activity were observed. While the SGPT levels did not vary significantly ($P<0.05$) among groups, fish in T_4 had considerably ($P<0.05$) low SGOT and ALP levels. In addition, dietary *M. sativa* treatment impacted serum creatinine levels. No significant variations were detected in the appearance of the intestinal mucosa or the shape of the villi. In *M. sativa* treated groups, serum bactericidal activity significantly ($P<0.05$) increased with the increase in extract concentration. The current findings clearly show that *M. sativa* possesses antioxidant and immune regulating properties, with no adverse effects on intestinal health, and might be a viable alternative to chemical medicines and antibiotics in aquaculture.

INTRODUCTION

Carps have long been the backbone of Indian fish farming, with the three principal endemic carps; namely, catla, rohu, and mrigal along with the exotic carps, silver carp, grass carp, and common carp; they contributing around 85% of the country's aquaculture production (**Ngasotter *et al*., 2020**). Common carp, *Cyprinus carpio* is one of the predominantly cultivated and economically important fish attributed to its exceptionally overwhelming nature, easy adaptation to artificial feed, fast growth and excellent feed conversion efficiency (**Khan *et al*., 2016**). It is critical to note that common carp is a versatile and opportunistic feeder that can shift from desired to optional feeding habits based on food availability (**Hoole *et al*., 2001**).

However, excessive rearing has resulted in the farming system being manipulated by adding chemical growth promoter for rapid growth in less time, which led to an immune loss and a disease outbreak. Notably, chemical treatments are being employed to address this issue, but their usage is complicated by the emergence of antibiotic-resistant bacterial strains, residual effects, and environmental degradation, among others.

Plant extracts has been emphatically proposed as a feed additive in aquaculture because of the secondary metabolites that have a wide range of medicinal activities, including immunostimulant, growth promoter, and antioxidant effects (**Zanuzzo** *et al.***, 2015**). Plants, in addition to beneficial factors, contain anti-nutritional factors that, when present in high concentrations, can be harmful to fish by altering the physiological balance and disrupting the lining of the intestine, which is critical for nutrient absorption and growth (**Prabu** *et al.***, 2017**). However, determining the correct dose to improve the immune system of fish while avoiding the potential for immunosuppression and intestine impairment is critical, thus diet must be modulated accordingly. Alfalfa, *Medicago sativa* is a plant containing a wide range of phytocompounds including saponins, flavonoids, phenolics, phytoestrogens, coumarins, alkaloids, amino acids, phytosterols, vitamins, digestive enzymes and terpenes (**Liu** *et al.***, 2018**). According to pharmacological reports, it is used to treat atherosclerosis, heart disease, stroke, cancer, diabetes; it is neuroprotective, hypocholesterolemic, antioxidant, antibacterial, hypolipidemic and estrogenic (**Bora & Sharma, 2011**). The current study is thus designed to investigate the potential impact of dietary alfalfa extract on the health status of common carp by evaluating hepato-renal marker enzyme activity, serum bactericidal activity and intestinal mucosal morphology.

MATERIALS AND METHODS

Feed formulation and proximate analysis

Five experimental diets (crude protein 38%) were prepared containing *Medicago sativa* extract at a rate of 0 (control), 2.5, 5.0, 7.5, 10 g kg^{-1} diets. All ingredients were collected from local market and grounded to powder. All the powdered ingredients were thoroughly mixed and appropriate volumes of water and fish oil were added to make a dough. Feed pellets were extruded from a manual pelletizer and dried in an oven at 50°C overnight. Dried pellets were then collected and stored in air- tight containers. Table (1) shows the ingredient and proximate composition of the experimental diets.

Table 1. Ingredient and proximate composition (% dry weight basis).

	T_0	T_1	T_2	T_3	T_4
Ingredient composition					
GNOC*	550	550	550	550	550
Soybean**	194	194	194	194	194
Fishmeal	124	124	124	124	124
Rice bran	61	60	58.5	57.5	56
Wheat flour	61	60	58.5	57.5	56
Mineral premix	10	10	10	10	10
***M. sativa* extract (g kg^{-1})**	0	2.5	5	7.5	10
Proximate composition					
Moisture %	7.72	7.10	8.62	9.11	7.52
Crude protein %	37.5	38.01	37.45	37.88	38.11
Crude fat %	3.58	2.69	3.87	3.63	3.54
Crude fiber %	8.66	8.27	6.87	7.23	8.27
Ash %	10.53	10.25	11.85	10.91	10.31
NFE* %**	32.01	33.68	31.34	31.24	32.25
Gross energy (kJ g^{-1})	15.77	15.83	15.76	15.75	15.94

*GNOC = groundnut oilcake, ***NFE = nitrogen free extract
Soybean was hydrothermally processed in an autoclave at 121°C (15 lbs for 15min) to eliminate antinutrient factors (Garg *et al*., 2002**).
All values are Mean ± SE of mean.

Experimental design

Cyprinus carpio fingerlings, with an average weight of 6.5 ± 0.3 g, were obtained from a local fish farm in Nilokheri, Kurukshetra, Haryana. and brought to laboratory in plastic bags filled with oxygenated water. Fish were initially fed the control food for 15 days to acclimatize to laboratory conditions before being distributed in 15 tanks at a stocking density of 12 fish per tank. Each group was allocated three tanks, which were hand fed the test meals twice a day for 60 days at a rate of 3% of the body weight. The photoperiod was maintained as 12h light and 12h darkness throughout the experimental trial. The average body weight of fish in each group was measured at interval of every 10 days for the whole experimental period.

Hepato-renal markers activity

At the end of the experimental trial, prior to blood sampling fish were fasted for 24h. Diluted clove oil (50 μl L^{-1}) was used to anesthetize the fish before blood sampling (**Ahmadifar *et al*., 2020**). Blood was drawn from the caudal vein with a 2ml BD syringe (Becton, Dickinson and Company) and 26-gauge needle, collected in a BD serum vacutainer, and incubated at room temperature for 2h to separate the serum. SGOT (Serum Glutamic-Oxaloacetic Transaminase), SGPT (Serum Glutamate Pyruvate Transaminase), ALP (Alkaline Phosphatase), and creatinine were measured using Reckon Diagnostics Pvt. Ltd. kits following the manufacturer's protocol.

Total proteolytic activity

Two fish from each tank were sampled for dissecting the entire digestive tract aseptically at 4°C. Samples were weighed, washed and rinsed with sterile saline solution (0.9 % NaCl), and then homogenized in a physiological saline solution, pH 7.4 (1:9 w/v) using an electric tissue homogenizer. The homogenate was centrifuged at $30,000 \times g$ for 30min at 4°C. The supernatant (crude extract) was collected and kept at -80°C until use. The total proteolytic activity was calculated using **Walter's (1984)** casein-hydrolysis technique as described by **Furne *et al.* (2005)** with slight modifications. Using various buffers, enzyme activity was tested at many pH levels in the digestive tract's physiological range. Glycine–HCl (pH 2.2), Acetate buffer (pH 4.6), Phosphate buffer (pH 7.4), glycine–NaOH (pH 9.2), and carbonate-bicarbonate buffer were utilised (pH 10.6). A standard curve was plotted using L-tyrosine. The amount of enzyme that released one mmol of tyrosine ml^{-1} min^{-1} was determined as one unit of total proteolytic activity.

Intestinal mucosal morphology

The intestinal samples were rinsed with PBS and promptly fixed with Karnovsky solution for primary fixation, washed with PBS (pH 7.4 and post-fixed for 2h at 4°C with 1% OsO_4 solution. The samples were subjected to dehydration in a graded level of alcohol, followed by a critical point drying with liquid CO_2. Tissue samples were then fixed on metal stubs, sputter-coated and examined for the appearance of intestinal mucosal folds and microvillus morphology using a scanning electron microscope (ZEISS-Evo 18) at 20 KX and 10 KX magnifications.

Serum bactericidal activity

Serum bactericidal activity was determined using the method of **Arulvasu *et al.* (2013)**. *Aeromonas hydrophila* (MTCC 1739) was obtained from IMTECH Chandigarh, cultivated for 24h at 30°C in nutrient broth media and harvested in PBS (pH 7.4). The bacterial culture was adjusted to achieve an optical density of 0.5 at 540nm, corresponding to 10^{-8} CFU ml^{-1}. Serum and bacterial suspension in equal amounts (100 μL) were mixed and incubated for 1h at 25°C. The serum was replaced with sterile PBS buffer to prepare a control. Afterward, the mixture was diluted (1:10) with sterile PBS. The serum bacterial mixture (100 μL) was pour-plated in nutrient agar and cultured for 24h at 37°C. Viable colonies were counted, and results were expressed as percentage of bactericidal activity.

Water quality parameters

To monitor and maintain the desired range, all physicochemical parameters of tank water were weekly examined, using the HANNA multiparameter HI-98194 kit following **APHA (1998)**.

Ethical clearance

The Panjab University's Institutional Animal Ethics Committee, Chandigarh, 160014, accepted and approved the research protocol with the number PU/45/99/CPCSEA/IAEC/2019/309.

Statistical analysis

An analysis of variance (ANOVA) followed by Duncan's multiple range test **(Duncan, 1955)** was performed in all groups to determine the significant variation between different treatments. The probability value ($P<0.05$) was chosen as the statistical significance level.

RESULTS

The mean final weight at the end of the experimental trial demonstrated that fish fed with more than 0.75% *M. sativa* extract supplemented feed gained significantly greater weight. (Fig. 1).
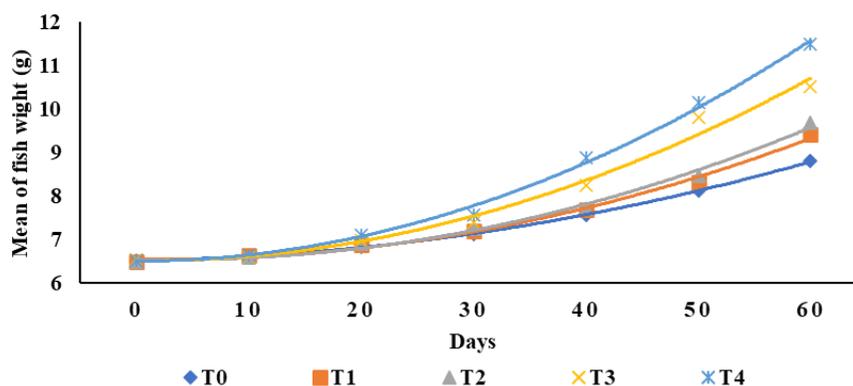

Fig. 1. Average mean weight of *Cyprinus carpio* fingerlings during experimental trial fed with dietary *Medicago sativa* extract supplemented diet at different doses (T_0 = control 0%, T_1 = 0.25%, T_2 = 0.5%, T_3 = 0.75% and T_4 = 1.0% extract).

Hepato-renal markers activity

No significant ($P<0.05$) difference was detected in SGPT levels between treatment groups after the feeding trial (Fig. 2a). However, increasing the concentration of dietary *M. sativa* extract in feed showed non-significant decreases in the level of SGOT till reaching 0.75% (Fig. 2b); while, after at 1% inclusion, a significantly low level of SGOT was observed. ALP level decreased significantly ($P<0.05$) upon increasing the dose of *M. sativa* extract (Fig. 2c). The serum creatinine level (Fig. 2d) also significantly ($P<0.05$) varied among groups as the dietary *M. sativa* extract supplemented fed groups showed low level compared to control (T_0).

Total proteolytic activity

The proteolytic activity increased in all groups from acidic to basic levels of pH range. Significantly ($P<0.05$), elevated proteolytic activity at all pH levels were observed in T_4, except at pH 2.2 where T_3 showed the highest activity, followed by T_4 compared to T_0 (Fig. 3). Significantly ($p<0.05$), the highest proteolytic activity in T_4 can be well reflected in terms of the highest weight gain in T_4, which attributes to the highest digestion of protein.
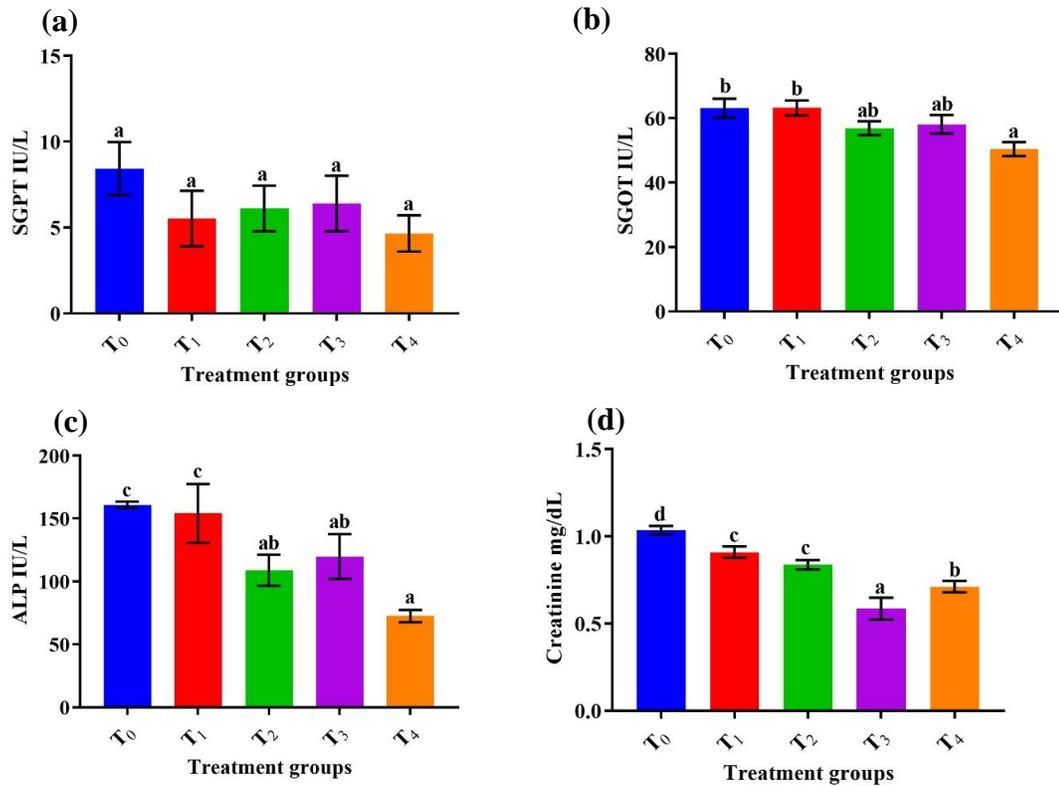
Fig. 2. Hepato-renal marker enzymes activity modulated by dietary *Medicago sativa* extract supplemented diet including (a) SGPT, (b) SGOT, (c) ALP and (d) creatinine.
Data in the bar graph represents mean ± SE of mean. All the data are analyzed by one- way ANOVA followed by Duncan's post hoc test. Different letters on bars represent significant differences ($P<0.05$).
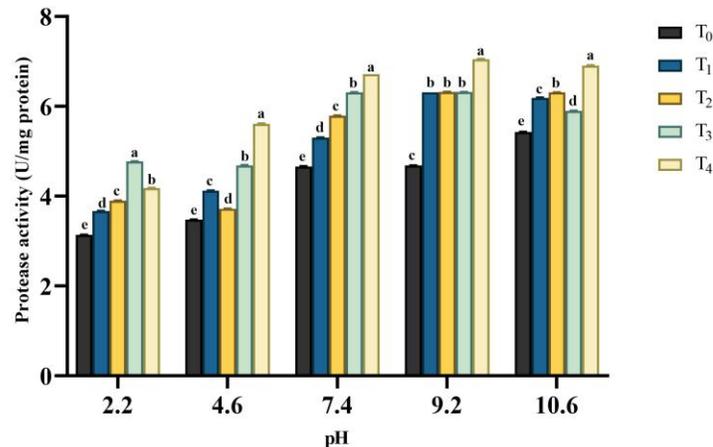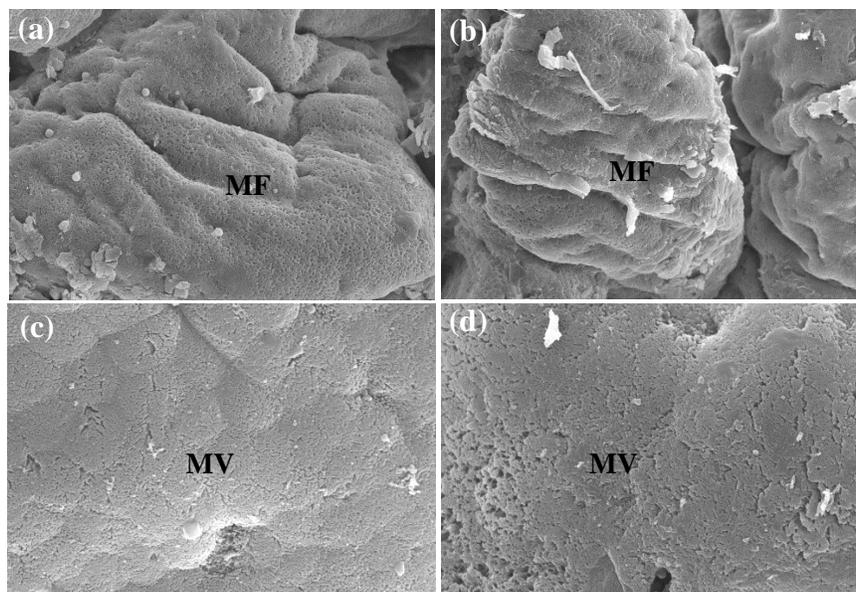

Fig. 3. Effect of dietary *Medicago sativa* extract supplemented diet on protease activity at different pH levels.
Data in the bar graph represents mean ± SE of mean. All the data are analysed by one- way ANOVA followed by Duncan's post hoc test. Different letters on bars represent significant differences ($P<0.05$).

Intestinal mucosal morphology

The mucosa of common carp intestinal tissue showed no significant variation in appearance in this study, although minor mucus secretion covering the mucosal surface in *M. sativa* treated groups (Figs. 4a, b) was observed, indicating that the plant extract has no detrimental effect on intestinal tissues. Furthermore, the luminal surface was covered by densely packed microvilli, with no significant difference ($P<0.05$) in the microvilli morphology observed (Figs. 4c, d).

Fig. 4. Scanning electron microscopy of intestinal tissue of common carp fed with dietary *Medicago sativa* extract supplemented diet. (a) Normal mucosal folds in control group and (b) normal mucosal folds with slight mucus droplets in *Medicago sativa* treated group and closely packed microvilli in control (c) and treated group (d). MF, mucosal folds; MV, microvilli. Scale bars 10 μm (a, b); 1μm (c, d).

Serum bactericidal activity

The serum bactericidal activity as calculated in terms of colony numbers in culture plates and the percentage bactericidal activity showed an exponential trend as the number of colonies decreased with increasing the dose of *M. sativa* extract in diet. While, the percentage of bactericidal activity increased with increasing dietary *M. sativa* concentration. T_4 had the highest percentage bactericidal activity ($P<0.05$) with fewest colonies (Figs. 5a, b).
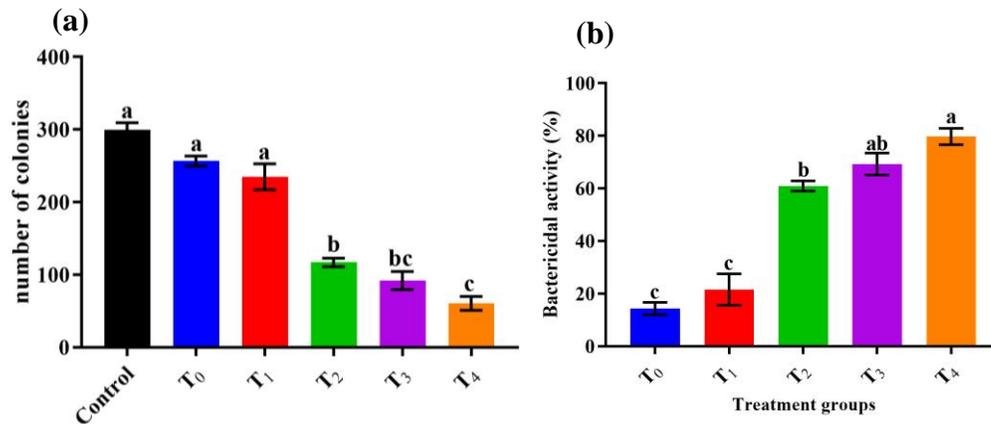
Fig. 5. Effect of dietary *Medicago sativa* extract on serum bactericidal activity in common carp shown as number of countable colonies (a) and percentage bactericidal activity (b)

Data in the bar graph represents mean ± SE of mean. All the data are analyzed by one- way ANOVA followed by Duncan's post hoc test. Different letters on bars represent significant differences ($P<0.05$).

Water quality parameters

Water physicochemical parameters were well maintained within the desirable range of common carp production during the feeding trial (Table 2).

Table 2. Effect of alfalfa extract supplementation in feed of *Cyprinus carpio* on physicochemical characteristics of water

Parameter	T_0	T_1	T_2	T_3	T_4
DO (mg L^{-1})	7.91 ± 0.008	7.33 ± 0.012	7.38 ± 0.014	7.28 ± 0.012	7.32 ± 0.014
pH	7.12 ± 0.008	7.52 ± 0.020	7.12 ± 0.037	7.41 ± 0.017	7.44 ± 0.030
Temperature (°C)	22.83 ± 0.13	22.63 ± 0.01	23.12 ± 0.39	22.61 ± 0.01	22.93 ± 0.04
TDS (ppm)	183.33 ± 2.40	193 ± 1.52	208.3 ± 1.45	188 ± 2.30	177.3 ± 2.40
Free CO_2 (ppm)	20.16 ± 0.03	20.6 ± 0.03	20.29 ± 0.04	20.42 ± 0.01	20.13 ± 0.02

DISCUSSION

Plant extracts, often known as phyto-biotics, have attracted a lot of interest as a possible alternative to antibiotics in aquaculture. Phytochemicals have been proven to enhance growth, stimulate appetite, improve immunity, and have stress-reduction, hepatoprotective, and anti-pathogenic properties in fish (**Yang *et al*., 2011; Reverter *et al*., 2014**).

The SGPT and SGOT enzymes are known as hepatic function indicators because their activity changes in fish exposed to relatively minor pollution or injury (**Machala *et al*., 1997**). The current study found that dietary *M. sativa* had no adverse effects on the health of common carp because the SGOT and SGPT levels were within the optimal range. The lower SGOT level in T_4 may be related to increased nutritional digestion and absorption efficiency, thus improved health status as reflected by the weight gain (Figure 1). Likewise, *C. macrocorpa* leaf essential oil (CMEO) to common carp (**Kesbiç *et al.,* 2020**), Silymarin treatment to rainbow trout (**Banaee *et al*., 2011**) or use of garlic and onion extract in catfish diet (**Al-Salahy, 2002**) reduced both SGOT and SGPT activities. The lower levels of ALP and creatinine in the *M. sativa* extract treated groups compared to the control groups in the current investigation showed inhibition of these enzymes as described in another study by **Kesbiç *et al.* (2020)**. The decreased level of serum ALP and creatinine suggests that dietary *M. sativa* extract supplemented diet did not have any harmful effects on the physiological status of common carp at the doses associated with high weight gain. ALP is a membrane-bound enzyme that participates in protein phosphorylation, cell proliferation, apoptosis, and cell migration (**Banaee *et al*., 2019**). Similar to our findings, **Soleimany *et al*. (2016)** discovered lower AST and ALP activity in the plasma of *Oncorhynchus mykiss* after consuming *Asparagus officinalis* extract for 15 days. Creatinine functions as a renal health indicator, and any injury to the kidney might cause their rise. Results of the present study revealed significantly ($p<0.05$) higher creatinine level in control group than the treatment groups suggesting an improved renal system performance in *M. sativa* extract treated groups attributed to various phytocompounds (**Em *et al*., 2015**). The effect of *M. sativa* extract on the activity of these enzymes may be due to flavonoid and antioxidant components in the extract. Excessive plant extracts can affect the liver function, increasing the activity of these enzymes due to the presence of anti-nutritional components as reported by **Parrino *et al.* (2019)** in rainbow trout which was not observed in this study.

Although the activity of each proteolytic enzyme can be evaluated using a specific approach, we followed **Furne *et al*. (2005)** and used a non-specific method. Several proteolytic activities were measured as a function of pH using this method. The low activity observed at acidic pH compared to neutral or basic pH could be due to cellular protease present in the homogenate (**Kuz'mina *et al*., 1990**). Similar to our findings, **Jonas *et al*. (1983)** found low activity of proteolytic enzyme in the digestive system of carp at acidic pH (2.0 and 4.0). Various authors have stated on occasion that the majority of Teleostei had higher protease activity at neutral and basic pH than acidic pH, which is consistent with our findings (**Overnell, 1973; Jany, 1976**).

In fish, intestine is the most important organ for digestion, absorption, and immunity. The integrity of morphology may represent the health state and absorptive function of the digestive tract. No injury or lesion caused by the dietary *M. sativa* extract in the intestinal mucosal lining was reported thus reflecting that it has no adverse effect

on the intestinal health **Meng *et al.* (2019)**. The presence of mucin droplets on the epithelial cells lends support to the idea that herbal extracts increase mucus secretion in the gastrointestinal tract. This effect minimises pathogen adhesion and helps to stabilise beneficial bacteria, protecting the intestinal villi and so increasing digestion and nutrient absorption **(Jamroz *et al*., 2006)**.

The improved serum bactericidal activity in groups fed *M. sativa* extract suggests that numerous humoral components involved in innate and/or adaptive immunity are boosted in the serum to effectively defend against infections. Similarly, *Achyranthes aspera* has enhanced serum bactericidal activity in *Labeo rohita* **(Rao *et al*., 2006)**.

CONCLUSION

The findings of current study indicates that the use of *M. sativa* extract as a dietary supplement in common carps feed had no adverse effects on the hepato-renal indicators and intestinal mucosal linings. Instead, including dietary *M. sativa* extract showed positive effects on common carp health in terms of final weight gain and serum bactericidal activity with increasing dosage of extract. As a result, it can be concluded that *M. sativa* extracts can be employed as a nutritional supplement in aquafeeds at an optimal dosage.

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