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Inclusion of Hot Water Extract of *Laurus nobilis* leaf in Koi carp Diet and its Impact on Growth Performance and Skin Mucosal Immunity

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Abstract

Koi carps are beautiful ornamental fish that are favoured for their stunning colours and beautiful appearance. In day-to-day life, ornamental fish culture is a much-preferred hobby by people, not only due to their attractive nature but also due to their stress-relieving properties. In this view, their growth enhancement and immune performance is of great concern. Phytobiotics and their usage in aquaculture sector has gained attention due to their versatile role as an appetite stimulator, growth enhancer and immune modulator. In this aspect, in the present study *L.nobilis* leaves was subjected to evaluation to explore their beneficial effects. In addition, they have sufficient, amount of Vitamin A, Vitamin B6, Iron, Potassium and calcium that may play a vital role in improving the immune status of fish. Further, they have certain enzymes that help in the digestion of food. In the present study, koi carp fed on the B2 diet registered a high production of 4.955 ± 0.06g compared to a low production of 2.289 ± 0.02g noticed in koi fed on the control diet. The skin mucosal immune parameters, such as total immunoglobulin, lysozyme activity, bactericidal activity and alkaline phosphatase, were high in fish received hot water extract (HWE) of *L.nobilis* leaf coated diet compared to control diet fed fish.

Introduction

Phytobiotics play a significant role in aquaculture industry replacing the ill effects of antibiotics. They enhance the quality of cultured fish in terms of growth promotion and immunity due to the presence of bioactive compounds like essential oils, alkaloids and flavonoids. Moreover, they stimulate the appetite and modify the gut microbial flora of fish thus exerting their positive effects. Recently, plant-based compounds have a prominent role in pharmaceutical, food and cosmetic industries. *L. nobilis* a perennial aromatic shrub, has notable therapeutic properties (Nava *et al.,* 2021). The leaves of this plant are known to possess antioxidant, anti-microbial and anticancer activities (Dobroslavic *et al*., 2021). Fish mucus has rich glycoproteins called mucins that play a crucial role in entrapping the pathogens, thus improving the health of the fish. The mucus of the fish consists of a consortium of immune factors that comprises of lysozymes, antimicrobial peptides, and immunoglobulins that directly lyse the pathogens upon contact with them. Further, alkaline phosphatase is a nonspecific enzyme that helps in detoxifying the proinflammatory microbial compounds. So, the evaluation of these factors in fish skin mucus provides valuable information about the immune status of the fish. Koi carps are beautiful ornamental fish that are grown in an aquarium for beauty, luck and prosperity, so the feed additives that improve their growth and health status are of prime importance. In this view, the present study was performed to test the effect of the hot water extract of *L.nobilis* leaf coated diet in the koi carp reared in indoor culture system.

Materials and methods

Koi carp

Koi carp was purchased from Jay Jay Aquarium, Nagercoil, Tamil Nadu. Only healthy and actively swimming fish were chosen for experimentation. The selected fish were packed in oxygenated polythene bags and transported to the culture site with minimal disturbance to reduce stress in the fish.

Hot water extract of *L. nobilis* leaf

L. nobilis leaf in a dried form was procured from a commercial market and used for the experimental purpose. The dried leaf was ground well in a mixer and about 10g of dried powder was mixed in 300ml of distilled water. The suspension was boiled for 3hrs and then filtered through nylon mesh and stored in appropriate container and this served as the hot water extract. Further, the extract was coated with koi carp diet at required concentration. Hot water extraction was based on the protocol of Fujiki *et al.,* 1992, with minor modifications.

Diet preparation

A commercial ornamental fish diet containing 35.0% crude protein was purchased from Jay Jay Aquarium, Nagercoil. The feed was segregated into four equal portions of 100 g each and then *L. nobilis* hot-water extract was incorporated into the diet at the levels of 2.0%, 5.0%, and 10.0%, and these diets were designated as B1, B2, and B3, respectively. In parallel, a control diet lacking *L.nobilis* extract was maintained for the analysis. HWE enriched *L. nobilis* diet was further dried in a hot air oven at 45º C to remove the moisture content.

Experimentation

Healthy koi carp weighing about 2.248 ±

0.02 g to 3.235 ± 0.06 g were segregated into respective control and experimental tanks (20 litre capacity) to initiate the experimentation. During the culture period, koi carp were fed on respective diets to fulfilment. The unfed remains were regularly siphoned out from the culture tanks, and fifty per cent of the water exchange was provided daily to maintain the optimum water quality parameters. Sufficient aeration was given in culture system to maintain optimum dissolved oxygen levels during the entire culture period using aqua air pumps (AP – 208).

Water quality analysis

The temperature in the culture tanks was analysed using a digital thermometer and the pH of the water samples was determined using a digital pH meter (Systronics) calibrated using standard buffers. The dissolved oxygen content of the culture system was analysed by Winkler's titration method (APHA 1995). Ammonia levels in the culture tanks were determined using the (API) Ammonia kit, and the colouration of ammonia levels was compared with the standard chart provided in the kit.

Growth Performance

Growth performance indices such as production, food consumption, FCR, FCE, SGR and G (%) were calculated based on the formulae given below.

Production

Production (g) = Final weight – Initial weight

Food consumption

Food consumption (g) = Food provided – Unfed remains

Food conversion ratio (FCR)

$$FCR = \frac{\text{Total amount of feed given (g)}}{\text{Total production of fish (g)}}$$

Food conversion efficiency (FCE)

$$FCE\ (\%) = \frac{\text{Wet weight of the fish produced (g)}}{\text{Dry weight of the feed given (g)}} \times 100$$

Specific growth rate (SGR)

$$SGR\ (\%) = \frac{\text{In final weight (g)- In Initial Weight (g)}}{\text{Experimental period}} \times 100$$

Growth Percentage (G %)

$$G\ (\%) = \frac{\text{Growth (g)}}{\text{Experimental duration}} \times 100$$

Evaluation of Skin Mucosal Immune Parameters

Mucus Collection

The skin mucus of koi carp was obtained by removing the mucus layer from the body surface using a sterile cell scraper. The collected samples were pooled, transferred into sterile eppendorf tubes, and maintained under chilled conditions in a thermocol box containing ice cubes for further analysis.

Total Immunoglobulin

Total immunoglobulin (TI) in the skin mucus of koi carp was determined according to the method of Siwicki and Anderson (1993) with minor modifications. Briefly, about 100µl of pooled skin mucus was treated with an equal amount of Polyethylene glycol (PEG) and centrifuged at 1000g for 10 minutes. The protein content of the skin mucus before the addition of PEG and the final supernatant was evaluated. The values were expressed in mg/ml concentration.

Total protein in mucus

$$\text{Total immunoglobulin (mg/ml)} = \frac{}{\text{Total protein in supernatant}}$$

Bactericidal activity

Bactericidal activity (BCA) in the skin mucus of koi carp was analysed by the MTT reduction principle based on the method of *Welker et al.,* (2007) with slight modifications. The colouration of formazan was read at a spectrophotometer at 560 nm that corresponds to the bactericidal activity. The results were expressed as percentage bactericidal activity.

Lysozyme Activity (LYZ)

Lysozyme activity was quantified in the pooled skin mucus of koi carp according to the protocol of Parry *et al.,* 1965, with minor modifications. A required amount of *Micrococcus lysodeikticus* (Sigma- M 3770, ATCC No 4698) was dissolved in sodium phosphate buffer. Then 1.8ml of buffer containing *M. lysodeikticus* was taken in a cuvette, and to this, 100µl of pooled skin mucus was added. The reduction in absorbance was read at 450 nm using Systronics spectrophotometer (Model 104) at regular time intervals, and finally the LYZ activity was expressed in U/ml.

Alkaline Phosphatase activity (ALP)

ALP activity was analysed in the pooled skin mucus of koi carp using the ERBA ALP kit. The ALP activity corresponds to the increase in optical density (OD), that was measured using a spectrophotometer at 405nm. The intensity of the yellow colour formed corresponds to the ALP activity.

Phytochemical analysis

Flavonoids

L.nobilis extract was treated with 5ml of dilute ammonia solution and to this concentrated sulphuric acid was added along the sides of test tube. Appearance of yellow colour indicated the presence of flavonoids.

Phenols

A required amount 1ml of extract was taken in a test tube and 2 ml of distilled water followed by a few drops of 10% ferric chloride was added. The appearance of blue or green colour indicated the presence of phenols.

Glycosides (cardiac glycosides)

A required amount (0.5ml) of plant extract was dissolved 2ml of glacial acetic acid, and to this, one drop of 5% ferric chloride solution was added. Finally, 1ml of con H_2SO_4 was added to the sides of the test tube. Appearance of brown ring at the interface of two liquid layers and the colour change in acetic acid layer to brown green indicates the presence of cardiac glycosides.

Terpenoids

L. nobilis extract 5ml was taken in a test tube at to this 2 ml of chloroform was added and to this concentrated sulphuric acid (3ml) was added carefully to form a layer. A reddish -brown colour at the interface indicated the presence of terpenoids.

Tannins

L. nobilis powder (dried form) 0.5g was taken in a test tube and boiled in 20ml of water in a test tube. The above mixture was filtered and finally a few drops of 0.1% ferric chloride were added. Appearance of brownish green colour indicated the presence of tannins.

Statistical Analysis

Statistical analysis was performed by One-way analysis of variance (ANOVA), followed by Duncan's multiple range test at $P < 0.05$ (Zar, 2009).

Table 1. Phytochemical constituents of *L.nobilis* leaves

Phytochemicals	Presence / Absence
Alkaloids	-
Flavanoids	-
Tannins	+
Terpenoids	+
Carbohydrates	-
Proteins	-
Phenols	+
Glycosides	-

Table 2. Water quality parameters in the culture tanks

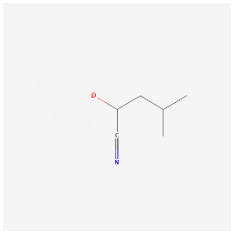
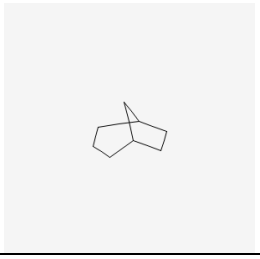
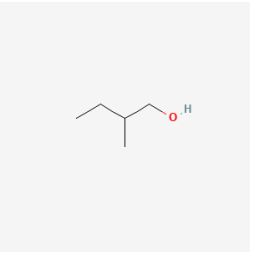
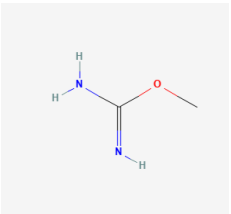
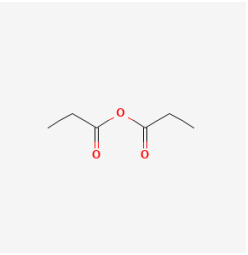
Parameters	Control	B1	B2	B3
Temperature (°c)	30.00 ± 0.04	30.00 ± 0.06	30.00 ± 0.04	30.00 ± 0.02
pH	7.74 ± 0.56	7.99 ± 0.42	7.90 ± 0.52	7.94 ± 0.64
Dissolved oxygen(mg/l)	5.82 ± 0.12	5.79 ± 0.17	5.64 ± 0.12	5.72 ± 0.14
Ammonia (mg/l)	< 0.1	< 0.1	< 0.1	< 0.1

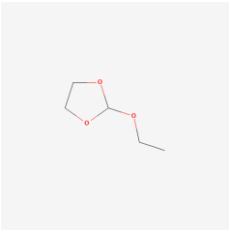
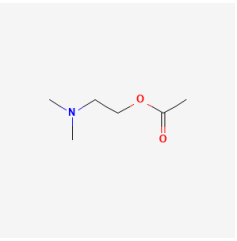
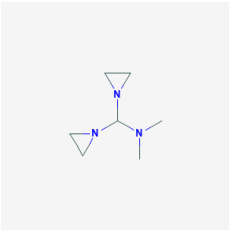
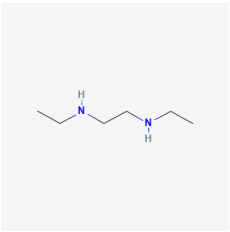
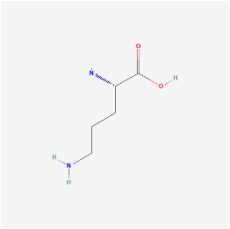
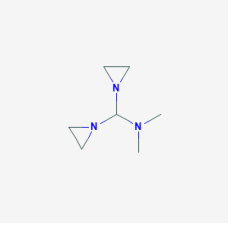
Table 3. Growth performance of koi carp fed on control and experimental diets

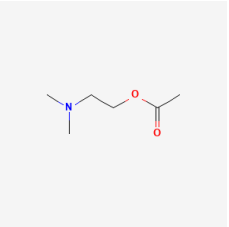
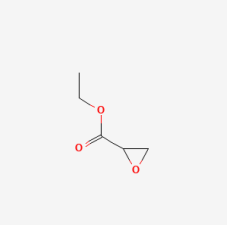
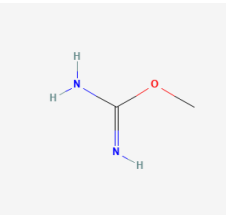
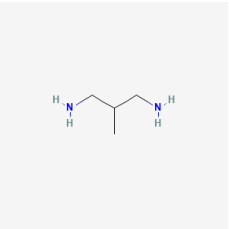
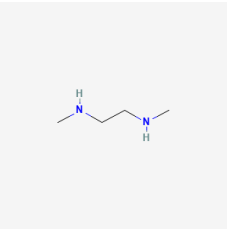
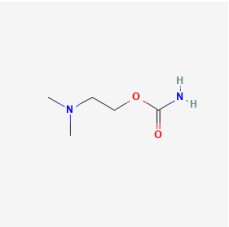
Parameters	C	B1	B2	B3
Initial weight (g)	2.403 ± 0.04	2.248 ± 0.02	2.933 ± 0.0.4	3.235 ± 0.06
Final weight (g)	4.692 ± 0.04	4.660 ± 0.04	7.888 ± 0.07	5.657 ±0.04
Production (g)	2.289 ± 0.02	2.412 ± 0.04	4.955 ± 0.06	2.422 ± 0.04
Food Consumed(g)	5.032 ± 0.04	5.043 ± 0.02	5.037 ± 0.02	5.042 ± 0.06
FCR	2.19 ± 0.02	2.09 ± 0.02	1.01 ± 0.03	2.08 ± 0.04
FCE (%)	45.48 ± 0.82	47.82 ± 0.94	98.37 ± 0.88	48.03 ± 0.90
SGR (%)	0.669 ± 0.02	0.729 ± 0.04	0.989 ± 0.02	0.558 ± 0.04
G (%)	5.72 ± 0.14	6.03 ± 0.13	12.38 ± 0.14	6.05 ± 0.12

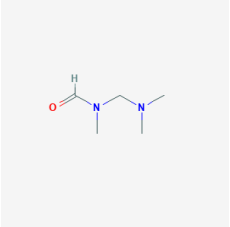
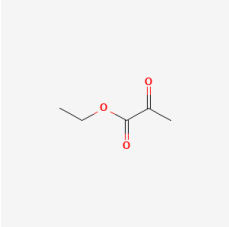
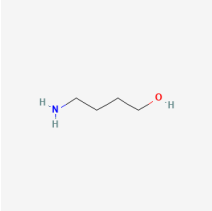
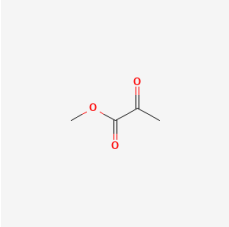
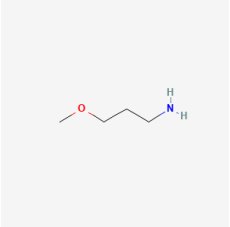
Table 4. GCMS analysis of *L.nobilis* leaf extract

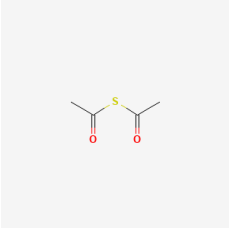
Name of the compound	RT	Area (%)	MW	Molecular formula	Molecular Structure

Pentanenitrile, 4-Methyl	17.209	16.829	97	C6H11N	
Bicyclo [3.2.1] octane	17.154	14.613	110	C8H14	
1-Butanol, 3-Methyl-	18.075	7.39	88	C5H12O	
O-Methylisourea Hydrogen Sulfate	13.828	6.052	74	C2H6ON2	
2-Butanone, 4-(Acetyloxy)-	15.589	5.013	130	C6H10O3	
2,2-Dimethyl-1, 3-Butanediol	17.835	4.772	118	C6H14O2	

1,2-Propanediol, 2-Acetate	13.858	4.429	118	C5H10O3	
Acetic Acid, 2-(Dimethyl amino) Ethyl Ester	22.106	4.269	131	C6H13O2N	
1,1-Bis [Aziridyltrimethylamine],	22.822	3.292	141	C7H15N3	
1,2-Ethanediamine, N, N'-Diethyl	20.291	3.126	116	C6H16N2	
Carbamic Acid,2-(DimethylAmino) Ethyl Ester	20.861	2.943	132	C5H12O2N2	
1,1-Bis [Aziridyltrimethylamine],	23.517	2.898	141	C7H15N3	

Acetic acid, 2-(Dimethylamino) Ethyl Ester	21.361	2.747	131	C6H13O2N	
1,2-Epoxy-3-Propyl Acetate	17.470	2.494	116	C5H8O3	
O-Methylisourea Hydrogen Sulfate	13.968	2.264	74	C2H6ON2	
1,3-Butanediamine	26.038	2.123	88	C4H12N2	
1,2-Ethanediamine,N,N'-Dimethyl-	24.197	1.686	88	C4H12N2	
Carbomic acid,2-(Dimethulamino) Ethyl Ester	20.591	1.519	132	C5H12O2N2	

N-Dimethylamino methyl-N-Methyl Formamide	18.860	1.371	116	C5H12ON2	
Propanoic Acide, 2-OXO-,Ethyl Ester	14.018	1.187	116	C5H8O3	
2-Propanamine, 1-Methoxy	24.858	1.164	89	C4H11ON	
Propanoic Acide, 2-OXO-, Methyl Ester	14.528	1.026	102	C4H6O3	
2-Propanamine, 1-Methoxy	25.483	0.924	89	C4H11ON	
N,N-Dimethyl-2-Amino Ethanol	20.351	0.839	89	C4H11ON	

Diacetyl Sulphide	14.073	0.766	118	C4H6O2S	

Results

Qualitative analysis of phytochemicals in HWE of *L.nobilis* leaves

The phytochemical analysis of the hot water extract of *L.nobils* leaves revealed the presence of tannins, terpenoids, and phenol whereas, alkaloids, flavonoids and glycosides were absent.

Water Quality Parameters

Water quality parameters monitored during the experimental duration is represented in Table 2. The temperature in the culture tanks showed mild fluctuations i.e. of 30.0 ± 0.02 ℃ to 30.0 ± 0.06 ℃ whereas, the pH and dissolved oxygen ranged between 7.74 ± 0.56 to 7.99 ± 0.42 and 5.64 ± 0.12 mg/l to 5.82 ± 0.12 mg/l in the culture tanks. Ammonia levels were negligible, i.e. < 0.1 mg/l in the culture tanks.

Growth performance

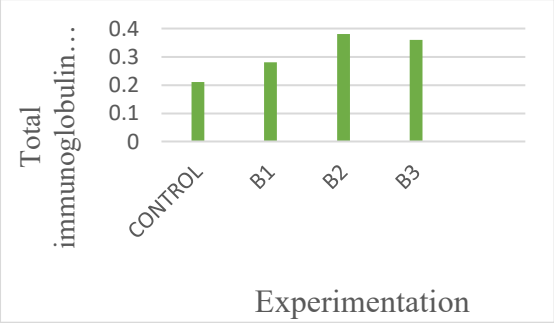
Growth performance of koi carp showed remarkable variations ($P < 0.05$) among the control and treated groups (B1-B3) as illustrated in Table 3. For instance, the production peaked high 4.955 ± 0.06g in B2 diet-fed fish compared to a low production of 2.289 ± 0.02g noticed in fish that received control diet. The specific growth rate SGR showed marked variations among B2- and control-diet-fed fish. For instance, a high SGR 0.989 ± 0.02 % was noticed in B2 diet-fed koi carp compared to a low SGR of 0.669 ± 0.02 % noticed in fish fed on control diet. The FCE food conversion efficiency peaked high 98.37 ± 0.88% in B2 diet fed fish whereas, in control, B1, and B3, it ranged from 45.48 ± 0.82%, 47.82 ± 0.94%, and 48.03 ± 0.90 %, respectively. The G (%) was remarkably high 12.38 ± 0.14 % in B2 diet-fed fish whereas, in control and other treated groups, it ranged from 5.72 ± 0.14 % to 6.05 ± 0.12 %, respectively suggesting that B2 concentration of HWE is the optimum dose that resulted in an increased weight gain.

Immunological parameters

Total immunoglobulin

Total immunoglobulin (TI) in the skin mucus of koi carp subjected to control, (B1-B3) diets showed marked variations ($P < 0.05$) among treatments. (Fig 1). For instance, the total immunoglobulin peaked high 0.38 ± 0.005 and 0.36 ± 0.002 mg/ml in B2 and B3 treated group compared to a low total immunoglobulin of 0.21 ± 0.004 mg/ml noticed in skin mucus of control diet fed fish. However, the total immunoglobulin showed higher value i.e. 0.28 ± 0.002 mg/ml in B1 diet fed fish compared to control fish.

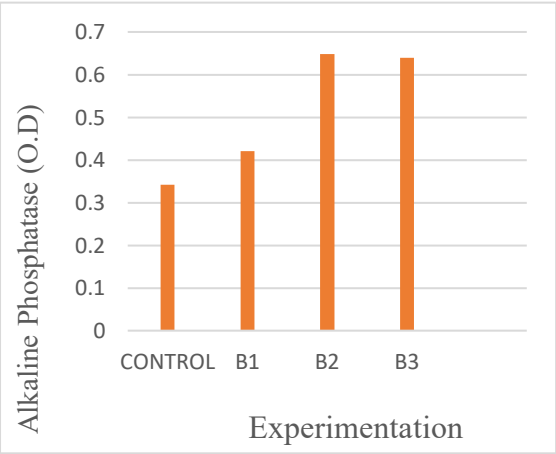
Fig1. Total immunoglobulin (TI) in the skin mucus of koi carp fed on control and experimental diets. (B1-B3)



Alkaline phosphatase

Alkaline phosphatase (ALP) recorded in the skin mucus of koi carp subjected to control and (B1-B3) treated diets is illustrated in fig 2. ALP values peaked high, i.e. 0.648 ± 0.003 O. D and 0.640 ± 0.005 O. D in B2 and B3 treated groups, whereas in B1 and control diet-fed fish, the ALP values declined i.e. 0.421 ± 0.004 O. D and 0.342 ± 0.006 O. D respectively.

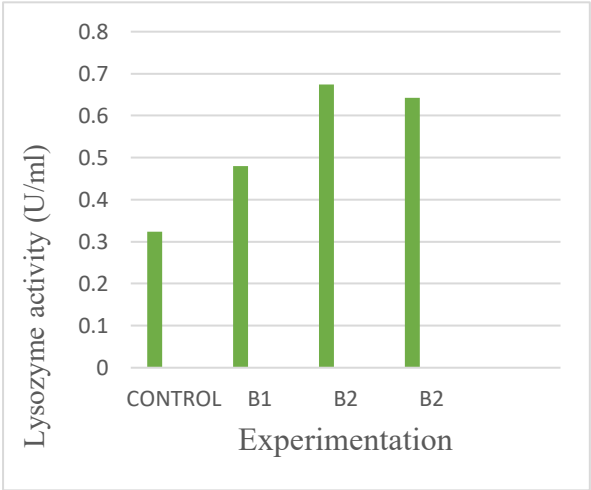
Fig 2. Alkaline Phosphatase (ALP) in the skin mucus of koi carp fed on control and experimental diets (B1-B3)



Lysozyme activity

Lysozyme activity (LYZ) in the skin mucus of koi carp showed fluctuations among control and treated groups (B1-B3) as illustrated in Fig 3. LYZ activity was remarkably high, 0.674 ± 0.004 U/ml and 0.642 ± 0.006 U/ml in the skin mucus of B2 and B3 treated groups whereas, LYZ activity declined, i.e., 0.480 ± 0.004 U/ml and 0.324 ± 0.002 U/ml in the skin mucus of B1 and control diet-fed fish.

Fig 3. Lysozyme activity in the skin mucus of koi carp fed on control and experimental diets (B1-B3)

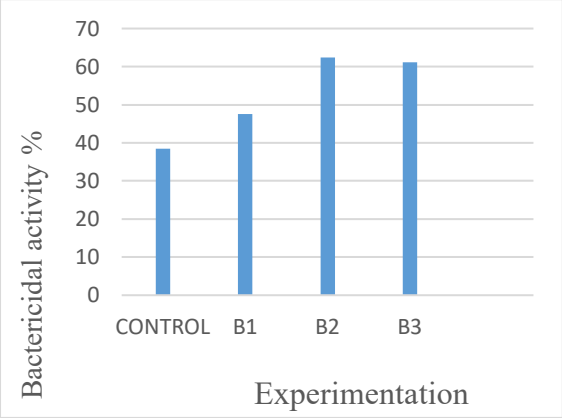


Bactericidal Activity

Bactericidal activity (BCA) in the skin mucus of koi carp registered notable variations ($P < 0.05$) among the control and treated groups (Fig.4). BCA activity ranged high 62.34 ± 0.72% and 61.20 ± 0.70% in the skin mucus of B2 and B3 diet-fed fish compared to a low BCA of 38.42 ± 0.52% observed in the skin mucus of control diet fed fish. However, B1 diet fed fish showed a higher BCA activity of 47.60 ± 0.64% in their skin mucus compared to fish those

received control diet.

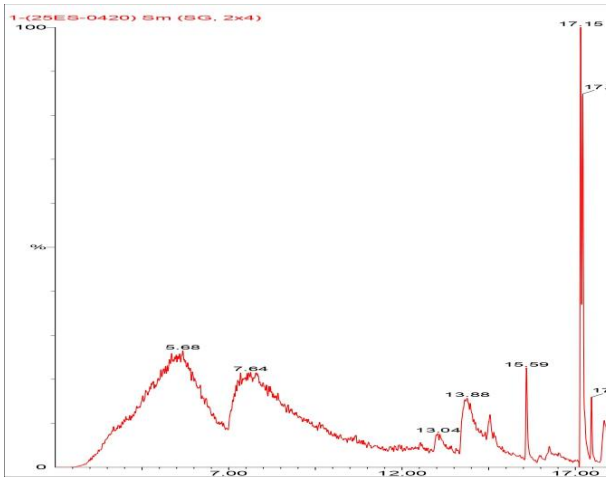
Fig 4. Bactericidal activity in the skin mucus of koi carp fed on control and (B1- B3) diets



GC-MS Analysis

GC-MS analysis of hot water extract of *L.nobilis* in represented in table 4. In total, 25 compounds made their presence of which pentane nitrile, 4-methyl 1 and Bicyclo (3.21 octane) were the major compounds. Other compounds such as 1-Butanol, 3-Methyl,0-Methyl lisource hydrogen sulfate 2-Butame, 2,2- Dimethyl-] ate were observed as minor compounds. The chromatogram is represented in Fig.5

Fig 5. Chromatogram of hot water extract of *L.nobilis* subjected to GCMS



Discussion

Phytobiotics considered as plant-based supplements and given to organisms to improve performance. Phytobiotics have a broad range of properties, such as: growth promoters, antioxidant, anticarcinogenic, antimicrobial, analgesic, antiparasitic and digestive enzyme function enhancement. In aquaculture, prophylactic administration of immunostimulants is among the most efficient methods of improving the defense system and control of diseases (Denev 2008). Concerning the growth-promoting properties, phytobiotics, stimulate the secretion of digestive enzymes that improve digestibility, leading to a better feed conversion and growth performance of the fish. In parallel to the above statement koi carp fed on B2 diet resulted in an improved FCE and an elevation in weight gain i.e. 98.37 ± 0.88% and 4.955 ± 0.06g in B2 diet fed fish compared to a low weight gain 2.281 ± 0.02g noticed in fish fed on control diet. *Mespilus germanica* leaf extract resulted in improved growth performance in *C. carpio* (Hoseinifar *et al* 2017).

Phytobiotics in the field of aquaculture have an immense role, such as appetite stimulation, growth-enhancing properties, aphrodisiac and immune enhancement. Inspite of these beneficial properties, they are also cost-effective and eco-friendly in nature (Citarasu 2010). So, in the present investigation, hot water extract *of L. nobilis* leaf was subjected to evaluation to observe its impact on koi carp reared in an indoor culture system by supplementing it as a feed additive at different doses. In the present study, Koi carp fed on B2 diet showed a significant increase ($P < 0.05$) in the bactericidal activity 62.34 ± 0.72% in their

skin mucus compared to a low BCA activity 38.42 ± 0.52% noticed in the skin mucus of control diet fish. The increase in BCA activity in the skin mucus of koi carp may be attributed to the bioactive compounds present in the extract of *L. nobilis* because there are sufficient evidence to show that plant extract possess bioactive compounds. Such as phenolics, proleoglycans, and flavonoids have the capacity to control pathogen. (Chakraborty and Hancz 2011). Further, evidence to support the BCA activity of plant extracts a few authors have reported that the methanolic extract of 31 Brazilian plants displayed a significance antibacterial activity against *A.hydrophila* , *Streptococcus agalactiae* and *Flavobacterium columnare* (Castro *et al*., 2008).

Phytochemicals are the plant components that play a critical role in disease prevention and antioxidant properties. In particular, tannins and saponins help to eliminate the microbes and also support wound healing. A variety of phytochemicals and belonging to several classes have rendered the inhibitory effects of microorganisms *invitro* (Cowan 1999). Likewise in the present study *L. nobilis* plant extract showed the presence of tannins that might have played in an important role in eliminating the pathogen especially *A. hydrophila* that could be evidenced from the increased BCA activity noticed in the skin mucus of B2 diet fed fish. The mucus secreted by the goblet cells holds a good proportion of glycoproteins typically lysozymes, lectins, and immunoglobins that play a versatile role in providing immunity to the candidate fish. So, in this aspect these factors are taken to evaluation.

L. nobilis leaf extract at B2 concentration showed a better lysozyme activity 0.674 ± 0.004 U/ml in their skin mucus in contrast to a low lysozyme activity of 0.324 ± 0.002U/ml noticed in the skin mucus of control diet fed fish. The increase in lysozyme activity can be correlated to the incorporation of the bioactive compounds present in *L.nobilis* leaf extract supplemented through the fish diet as stated by Christaki *et al.,* 2020. There are reports by several researchers that dietary plant extract improves the mucosal immune response in fish (Hoseinifar *et al.,* 2015, Giri *et al.,* 2019; Giri *et al*., 2002)

Immunoglobin in the skin mucus play a vital role in neutralising the pathogens and an increase in the Ig levels shows a better immune function (Parra *et al*., 2015). The present results evidenced an enhanced total immunoglobin in the skin mucus of fish supplemented with B2 diet whereas, the total immunoglobin in fish those fed on control diet exhibited a decreased value for TI. However, the type of plant extract and the dose of the extract will influence the growth depending upon the fish species (Yilmaz *et al.,* 2023). Similarly, in the present study *L.nobilis* leaf extract at B2 concentration resulted in a significant increase ($P < 0.05$) in weight gain compared to control group. Alkaline phosphatase (ALP) plays a significant role in fish immunity during the early stages of wound healing. Likewise, in the present investigation a high ALP activity of 0.648 ± 0.003 O.D was noticed in the skin mucus of B2 treated group whereas, a low ALP activity of 0.342 ± 0.006 O. D was noticed in the skin mucus of koi carp fed on control diet.

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