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Quantification of phytochemicals in Muli bamboo [*Melocanna baccifera* (Roxb.) Kurz] leaf and shoot extracts and immunomodulatory effect of dietary ethanolic leaves extract on *Labeo rohita* (Hamilton)

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Traditionally, bamboo has immense medicinal values owing to their rich bioactive compounds. On the other hand, scientific developments in global aquaculture have revealed the significance of dietary nutraceuticals in aquaculture. In this study, efforts have been made to evaluate the properties of shoot and leaf extracts of *Melocanna baccifera* (Roxb.) Kurz (Muli bamboo) and demonstrated its immunomodulatory potential. At first, four different extracts of bamboo leaf and shoot were prepared through water and ethanolic solvents followed by phytochemical profiling, antioxidant and antimicrobial activities of extracts. Based on the *in vitro* evaluation, BLAL (bamboo leaf alcoholic) extract was chosen for further *in vivo* evaluation. Second experiment was carried to assess the toxicity of BLAL extract on the Indian major carp, *Labeo rohita* (Hamilton), locally called 'rohu'. No mortality was observed up to 20 g of extract kg^{-1} body weight. Additionally, haemolytic assay was also conducted to ascertain the cellular toxicity of extract. Third experiment was conducted to find out the effect of dietary BLAL extract [doses: control (0.0%), T1 (0.01%), T2 (0.1%) and T3 (1%) of extract kg^{-1} feed] on immune related genes (*HSP70*, *IL-1β* and *TNF-α*) expression in *L. rohita*. The present study confirms the presence of vital phytochemicals in bamboo extracts and immunomodulatory potential of ethanolic leaf extract in rohu.

Keywords: Antimicrobial, Antioxidant, Carp, Immune related genes, Rohu

Recent global scenario is shifting from food security to nutritional security and aquaculture serves as a liable and sustainable option to the concern. Over the years, aquaculture sector have emerged as a flourishing sector as one of the world's fastest growing sector among all agri-based commodity[1]. Here, fish imparts the majority of share and serves as an excellent source of superior and easily digestible protein with the optimal blend of essential fatty acids such as omega-3 fatty acids and other nutrients such as vitamins (A and D), minerals (selenium, calcium, phosphorus, sulfur), etc.[2]. On the other hand, phytochemicals/ botanicals are commonly found in plants, referred as a diverse group of phytogenic active compounds which biosynthetically originate from primary metabolites[3], which includes flavonoids, alkaloids, phenolics, ascorbic acid, saponin, rotenone, terpenoids, steroids, essential oils, etc. These secondary metabolites are having immense potential to be used as pharmacological entities and they can be an excellent ecofriendly alternative for chemotherapeutics without or with very minimal toxic effect. Because of their unparallel attributes, presently phytochemicals are beheld as a replacement for chemical remedial agents such as antibiotics, anabolic growth promoters, immunoboosters, etc[4].

The rationale behind the presence of a diverse variety of phytochemicals and non-nutritional compounds in the plants is a result of evolution over the time through which plants have acquired heightened defence against various threats like microorganisms, pests and environmental stress, etc[5,6]. Among the various medicinal herbs, bamboos have a long history of medicinal importance from the ancient era such as antiinflammatory, antioxidant, antihelmintic, anticancer, antimutagenic, antiallergic, antihypertensive, antifungal, antibacterial, antiviral, immunostimulant, prebiotic, etc[7-11]. Despite all these vital attributes, the application of bamboo and its derivatives in agriculture and allied sector such as aquaculture is still underexplored. Therefore, in the present study, we envisaged to harness the array of phytoconstituents present in the bamboo and to

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examine the immunomodulatory potential of extract on rohu (*Labeo rohita*) up on dietary administration.

Materials and Methods

Preparation of extract

Most abundant bamboo variety in the entire northeast India, *i.e.*, Muli bamboo [*Melocanna baccifera* (Roxb.) Kurz] was chosen for the present study[11]. Bamboo shoot and leaves for the extraction were collected from the local forest of Lembucherra, Agartala, India (latitude: 23.829321 and longitude: 91.277847). Validation of the identified species was done with the help of Prof. Badal Kumar Datta, Professor and HOD, Department of Botany, Tripura University, Agartala and further verified at http://www.theplantlist.org.

Extraction of active compounds from bamboo parts was done through two solvents *i.e.*, nonpolar (90% ethanol) and polar (water)[11]. At First, leaves and shoot were shade dried and then grounded. Grounded samples were soaked separately in a ratio of 10:1 and kept in a shaker for 48 h. Later, the soaked samples were centrifuged at 2460×*g* for 10 min and then filtered through Whatman filter paper (No.1). Collected supernatant was evaporated in the rotary evaporator at a constant temperature of 40°C and later freeze-dried. The collected extracts were kept in –20°C until further use. The yield percentage of the extract was calculated as: weight of extract × 100/weight of dry sample.

Property estimation of extracts (1st experiment)

For characterization of extracts, stock solution for phytochemical and antioxidant evaluation was prepared at a uniform concentration of 1.0 mg of the extract in one mL of absolute ethanol. Whereas for antimicrobial assay, 10 mg of the extract was dissolved in one of mL of DMSO (dimethyl sulfoxide).

Phytochemical profiling

Determination of alkaloid was done by following the method of Mir *et al.*[12], where percentage of total alkaloid content was calculated as: weight of residue × 100/weight of sample taken. Total phenolic content was determined by following the Siddhuraju & Becker[13] method and represented as mg of gallic acid equivalent (mg GAE g^{-1} of the extract) using the standard calibration curve of gallic acid. For evaluation of total flavonoids content, Jia *et al.*[14] method was followed and expressed as mg QE g^{-1} (mg quercetin equivalent g^{-1} of the extract) using the standard calibration curve of quercetin. Ascorbic acid content was evaluated through Klein & Perry[15] method and content was expressed as mg of ascorbic acid equivalent (mg AE g^{-1} of the extract) using the calibration curve of L-ascorbic acid. Whereas, total saponin content was determined by following the Makkar *et al.*[16] method, where saponin content was presented as mg of diosgenin equivalents (mg DE g^{-1} of the extract) using a standard calibration curve of diosgenin. Rotenone and tannin content of extract were also determined by the method of Pagan[17] and Schanderl[18], respectively.

Antioxidant assay

DPPH (1,1-Diphenyl-2-picrylhydrazyl) activity was determined as described by Brand *et al.*[19] and scavenging percentage was calculated as: 100 - [absorbance (sample) - absorbance (blank) × 100/absorbance (control)]. Hydrogen peroxide (H_2O_2) radical scavenging activity was assayed using the method given by Ruch *et al.*[20], where percentage scavenging activity was calculated using: absorbance (control) - absorbance (sample) / absorbance (control) × 100. Whereas, Ruch *et al.*[20] method was followed for alkaline DMSO radical scavenging activity and percentage scavenging activity was calculated using the equation: absorbance (control) - absorbance (sample)/absorbance (control) × 100.

Antimicrobial test

Pure bacterial (*Aeromonas hydrophila*, *Edwardsiella tarda* and *Escherichia coli*) and fungal culture (*Saprolegnia parasitica* and *Aphanomyces invadans*) were collected from the Department of Aquatic Health & Environment, and Department of Fish Processing Technology & Engineering, College of Fisheries, CAU, Tripura[11,21,22] and *Aspergillus oryzae* ATCC 10124 was procured from HiMedia, Mumbai. Standard methods were followed for conducting the antimicrobial sensitivity test[23].

Prior to use, the microorganisms were allowed to grow in their respective selective media or nutrient agar, as per need. For bacterial sensitivity test, about 100 µL of broth were spread on solidified agar plates. Whereas for the fungal sensitivity test, about 100 µL of spores were spread on GP (glucose-peptone) agar plates followed by well (6 mm) preparation on both the plates with the help of gel puncture. The respective extracts were poured in the wells along with control and blank. Bacterial plates were incubated for 37°C for 48 h whereas fungal plates at 20°C for 48 h. After the incubation, the inhibition

zones were recorded. Penicillin and streptomycin antibiotic mixture (HiMedia, India) and ketoconazole (Sigma Aldrich, USA) were used as the positive control for the antibacterial and antifungal test, respectively whereas DMSO was used as a negative control in both the tests.

Collection and maintenance of fish

Healthy *L. rohita* fingerlings (average length 13.7±1.5 cm and weight 18.2±1.5 g) were collected from the farm of College of Fisheries, Lembucherra and they were acclimatized for about 15 days in fibre reinforced plastic (FRP) tanks of 500 L capacity. Fish were kept in the FRP tanks with the routine observation and fed with the pelleted feed twice a day @ 2% of their body weight.

Toxicity test (2nd experiment)

Haemolytic assay

The haemolytic activity was performed in 96-well microtiter plate by following the method of Paniprasad and Venkateshvaran[24] with some modification. About 100 µL crude extract in PBS (5 mg mL^{-1}) and same volume of erythrocyte suspension was added to wells. Distilled water and normal saline were used as a positive and negative control in the assay, respectively. The lytic activity of extract was expressed as a haemolytic unit (HU). The dilution, which showed the complete haemolysis, was taken as one hemolytic unit (HU).

Acute toxicity assay

Toxicity test was conducted to determine the effect of BLAL extract on *L. rohita* fingerlings where fish were fed with BLAL extract at variable concentration (2.5, 5, 10 and 20 g kg^{-1} of body weight). Mortality/ morbidity of fingerlings were observed in every 6 h up to 96 h. All these tests were conducted in glass aquariums using triplicates with 5 fingerlings per aquaria.

Effect of BLAL extract on rohu (3rd experiment)

Preparation of diets

Four purified diets [control (0.0%), diet T1 (0.01%), diet T2 (0.1%) and diet T3 (1%) of the extract Kg^{-1} feed) were prepared by incorporating the calculated amount of BLAL extract[11]. Ingredients required for the formulation of the feed mentioned in Table 1.

Experimental design

Tests were carried out in three replicates, each test group comprising 30 fingerlings in 500 L circular FRP tanks. Altogether, 360 numbers of *L. rohita* fingerlings were randomly distributed in twelve FRP tanks following CRD (completely randomized design). The fish were divided into four groups (Control, T1, T2 and T3). The control diet was devoid of extract. The remaining groups T1, T2 and T3 were fed with feed containing 0.01, 0.1 and 1% of BLAL extract, respectively. Feeding was done twice a day at satiation level up to 60 days. Samples for immune related gene expression analyses were collected on last day of experiment (60th day).

Table 1 — Formulation of experimental diets (100 g)

Ingredients	Diet Quantity (in g)			
	Control	T1	T2	T3
Casein	39.0	39.0	39.0	39.0
Gelatin	8.0	8.0	8.0	8.0
Dextrin	31.0	31.0	31.0	31.0
CMC	2.0	2.0	2.0	2.0
Veg. oil	3.0	3.0	3.0	3.0
Cod liver oil	3.0	3.0	3.0	3.0
Vit. mixture*	6.0	6.0	6.0	6.0
BHT	0.2	0.2	0.2	0.2
Betain	0.2	0.2	0.2	0.2
Cellulose	7.7	7.69	7.6	6.7
Extract (BLAL)	0.0	0.01	0.1	1.0
Total	100	100	100	100
Proximate composition of diets				
Moisture	10.14±0.11	10.16±0.12	9.98±0.12	10.11±0.14
Crude protein	35.85±0.1	35.95±0.1	36.38±0.1	36.65±0.2
Total lipid	2.04±0.5	2.05±0.7	2.06±0.9	2.07±0.1
Ash	19.81±0.04	19.34±0.01	19.41±0.03	19.03±0.01
Carbohydrate	32.17±0.24	32.50±0.14	32.16±0.17	32.92±0.12

[*Composition of vitamin-mineral premix: Vit. A, 5500000 IU; D3, 1100000 IU; B2, 2000 mg; E, 750 mg; K, 1000 mg; B6, 1000 mg; B12, 6 mcg; calcium pantothenate, 2500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg; L, lysine 10 g; DL, methionine 10 g; and selenium 50 mg l^{-1}]

Immune gene expression analyses

Tissue samples (liver and head kidney) were collected (pooled for each replicates) and stored in TRIzol (Invitrogen, USA) and kept in −80°C. Three immune genes (*HSP70*, *IL–1β* and *TNF–α*) were chosen for the present transcriptional analyses and the relative expression was compared with the help of a housekeeping gene, 18S rRNA (Table 2).

TRIzol method was used for the isolation of total RNA from the immunocompetent tissue. The concentration of isolated RNA was determined with the help of spectrophotometer (Eppendorf Bio-Spectrometer, USA) and the integrity of the extracted RNA was determined through agarose gel electrophoresis. Followed by, DNase treatment to remove any DNA contamination. The mRNA transcript of the genes was converted into complementary DNA using the first-strand cDNA

Table 2 — Primers used in immune gene expression analyses

Target gene	Primer sequence (5' to 3') Forward/ Reverse	Annealing temp. (°C)	Accession No.
HSP70	CTGTACGAGGGCATCGACTT GTCCATCTTGGCGTCTCTCA	60	KM369886
IL–1β	GGTGCTGAGCGACGAACTAA GTTGGCCGATGTTTTGTGGG	60	AM932525
TNF–α	AGGCGGCTTGAAAGTAGTGG TATGCAGAACGTCGTGGTCC	60	FN543477
18S rRNA	TCGCTAGTTGGCATCGTTTATG CGGAGGTTCGAAGACGATCA	60	GU967674

synthesis kit (Fermentas, USA) as per the protocol supplied by the manufacturer. The resulting cDNA was used for PCR and the remaining cDNA was stored at –80°C for further use. Generated cDNA was used as a template for quantitative PCR through SYBR green method. To rule out any non-specific amplification, melt curve analysis was performed for each sample. The relative expression of immune genes was compared with constitutively expressed 18S rRNA gene as a reference transcript. Each sample was analyzed in triplicate and the total reaction mixture was optimized in 10.0 µL of reaction volume (Table 3). All the standard guidelines recommended by institute were followed during all the experiments.

Statistical analysis

SPSS (SPSS Inc., Chicago IL, USA) was used for statistical analysis of the data. Results were represented as a mean ± standard error (SE). Comparative analysis of mean values was determined using one-way ANOVA and Duncan's test. Probability levels of 0.05 were used to find out the significance in various cases.

Results

Preparation of extract

The extraction capacity of both the solvents revealed that the ethanolic solvent was much efficient in yielding the phytoconstituents as compared to aqeous solvent for both bamboo shoot and leaf (Table 4).

Phytochemical profiling

Alkaloid content of BSAQ extract was significantly (P <0.05) high than all other extracts. There was no significant (P >0.05) difference between the extract for total phenolic content (Table 5). However, the BSAL extract showed maximum content of phenolic compounds among all the extracts. BSAL extract showed significantly (P <0.05) high content of flavonoids among all other extracts. Ascorbic acid content of BLAL extract was significantly (P <0.05) high as compared to other three extracts. BSAL

Table 3 — Composition of PCR reaction mixture

Components	Concentration	Volume (µL)
cDNA template	2.0 ng	1.0
Master mix	1X	5.0
Primer Forward	1 pmole	1.0
Primer Reverse	1 pmole	1.0
MQ water	-	2.0

Table 4 — Yield percentage for different types of extracts

Extract	Yield (%)
BSAQ (Bamboo Shoot Aqueous) extract	28.81
BSAL (Bamboo Shoot Alcoholic) extract	29.27
BLAQ (Bamboo Leaf Aqueous) extract	30.14
BLAL (Bamboo Leaf Alcoholic) extract	30.79

showed significantly (P <0.05) higher level of saponin as compared to other extracts. Rotenone content of BLAL was significantly (P <0.05) high compared to other remaining extracts. Whereas, tannin content of BLAQ extract was significantly (P <0.05) high compared to BLAL, BSAQ and BSAL extract.

Antioxidant assay

DPPH radical scavenging activity of BLAL extract was significantly (P <0.05) higher than the other three remaining extracts. BLAQ extract showed significantly (P <0.05) higher level of H_2O_2 radical scavenging activity as compared to other extracts. Whereas, alkaline DMSO radical scavenging response of both BLAQ and BLAL extracts were significantly (P <0.05) higher than the reamining extracts.

Antimicrobial tests

The results of the antimicrobial tests have been shown in Fig. 1 with all the extracts depicted variable levels of inhibitory response against both bacterial as well as fungal test organisms.

Haemolytic assay

Haemolytic activity (diffused appearance) of the extracts was compared with non-haemolytic PBS control (button shape at the bottom). Lysis caused by the extracts on chicken blood was represented by HU, and assay revealed haemolysis for all four extracts. The lytic responses (HU) were graded as 4, 4, 2 and 4 for BSAQ, BSAL, BLAQ and BLAL extract, respectively.

Acute toxicity assay

Based on the results of *in vitro* tests, the BLAL (bamboo leaf alcoholic) extract was found to be the best extract in terms of cumulative score based on phytochemical, antioxidant and antimicrobial attributes, and chosen for further *in vivo* evaluation (Table 6). Under this scoring system, minimum one to

Table 5 — Phytochemicals and antioxidant properties of extracts*

Type of extract	Content/Percentage
Alkaloid percentage	
BSAQ	27.27±0.58[a]
BSAL	10.56±0.38[d]
BLAQ	25.25±0.55[b]
BLAL	15.18±0.25[c]
Phenolic content (mg GAE g^{-1} of extract)	
BSAQ	32.1±1.92[ab]
BSAL	41.52±3.76[a]
BLAQ	29.2±3.16[b]
BLAL	32.1±2.90[ab]
Flavonoids content (mg QE g^{-1} of extract)	
BSAQ	70.0 ±1.10[b]
BSAL	144.19±1.80[a]
BLAQ	14.82±0.36[d]
BLAL	56.69±0.95[c]
Ascorbic acid content (mg AE g^{-1} of extract)	
BSAQ	2.1±0.21[b]
BSAL	3.36±0.55[b]
BLAQ	2.81±0.44[b]
BLAL	7.57±0.21[a]
Saponin content (mg DE g^{-1} of extract)	
BSAQ	217±2.31[c]
BSAL	337±2.31[a]
BLAQ	220.34±0.67[c]
BLAL	285±3.06[b]
Rotenone content (mg RE g^{-1} of extract)	
BSAQ	3.43±0.11[a]
BSAL	5.23±0.11[b]
BLAQ	6.22±0.10[c]
BLAL	7.46±0.011[d]
Tannin content (TAE g^{-1} of extract)	
BSAQ	2.29±0.06[a]
BSAL	2.01±0.06[a]
BLAQ	3.6±0.3[b]
BLAL	3.11±0.07[c]
DPPH radical scavenging activity (%)	
BSAQ	67.72±2.83[bc]
BSAL	69.89±0.99[b]
BLAQ	62.75±0.37[c]
BLAL	80.24±1.50[a]
H_2O_2 radical scavenging activity (%)	
BSAQ	93.7 ±0.28[b]
BSAL	90.81±0.33[d]
BLAQ	95.38±0.08[a]
BLAL	92.75±0.45[c]
Alkaline DMSO radical scavenging activity (%)	
BSAQ	65.46±0.52[c]
BSAL	69.16±0.59[b]
BLAQ	83.36±0.41[a]
BLAL	82.87±0.34[a]

[*Values are presented as mean ± SE. Significant differences (P <0.05) are indicated by different superscript (a, b, c and d)]

maximum four score was allotted to the extract based on their respective properties *i.e.*, score four was awarded to the extracts which exhibit maximum phytochemical content/ inhibition zone/ radical scavenging activity as compared to other extracts and subsequently the score of three, two and one were awarded to the remaining extracts concerning their properties. Based on cumulative score, BLAL extract was chosen. In acute toxicity evaluation, healthy fingerlings of *L. rohita* were exposed to a series of BLAL extract incorporated feed with 2.5, 5, 10 and 20 g kg^{-1} of body weight. Among all these doses, no mortality was observed up to 96 h of observation. However, at the maximum dose, rohu fingerlings became lethargic but recovered within 6 h (approximately).

Table 6 — Characterization scorecard of all four extracts*

Parameter/Microorganism	BSAQ extract	BSAL extract	BLAQ extract	BLAL extract
Alkaloid content	4	1	3	2
Total phenolic content	3	4	2	3
Total flavonoids content	3	4	1	2
Ascorbic acid content	1	3	2	4
Saponin content	1	4	2	3
Rotenone content	1	2	3	4
Tannin content	2	1	4	3
DPPH activity	2	3	1	4
H_2O_2 radical scavenging activity	3	1	4	2
Alkaline DMSO radical scavenging activity	3	1	4	2
Saprolegnia parasitica	4	3	3	2
Aspergillus oryzae	1	2	4	3
Aphanomyces invadans	3	4	2	2
Aeromonas hydrophila	3	1	4	2
Edwardsiella tarda	2	3	1	4
Escherichia coli	1	4	3	3
Total score	37/64	41/64	43/64	45/64

[*Scores of minimum 1 to maximum 4 was allotted based on their respective properties]

Immune gene expression analysis

HSP70, *IL-1β* and *TNF-α* expression in both liver and head kidney tissues were significantly ($p < 0.05$) high in T1, T2 and T3 as compared to control as shown in Fig. 2 (A-F).

Discussion

In the present study, overall percentage yield for both bamboo leaf and shoot were high for the alcoholic solvent compared to the aqueous solvent. It may be because of the higher efficiency of alcoholic solvent for drawing out wide variety of active constituents which is corroborated by several other reports suggesting that the combined use of water and alcohol (for harnessing the ability of both solvents polar and non-polar) assisted in higher extraction efficiency [25,26]. This could be the possible reason for higher yields of hydroalcoholic extracts compared to the water solvent. The yield (extraction efficiency) also depends upon the factors like nature of solvent (polar or non-polar), nature of the phytochemical,

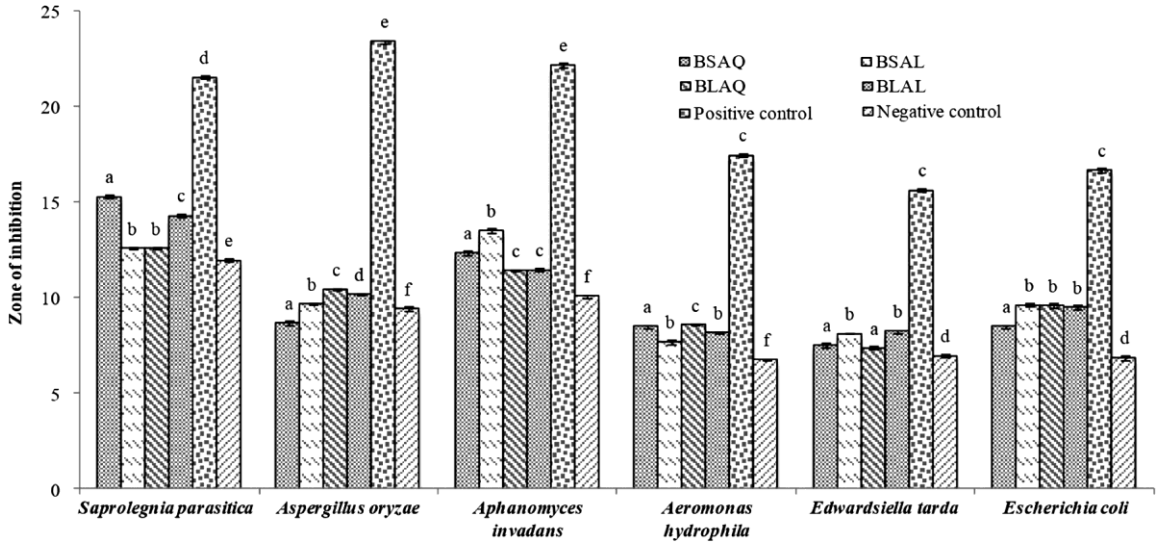

Fig. 1 — Zone of inhibition (in mm). [Values are presented as mean ± SE. Significant differences (P <0.05) are indicated by differe superscript (a, b, c, d, e and f)]

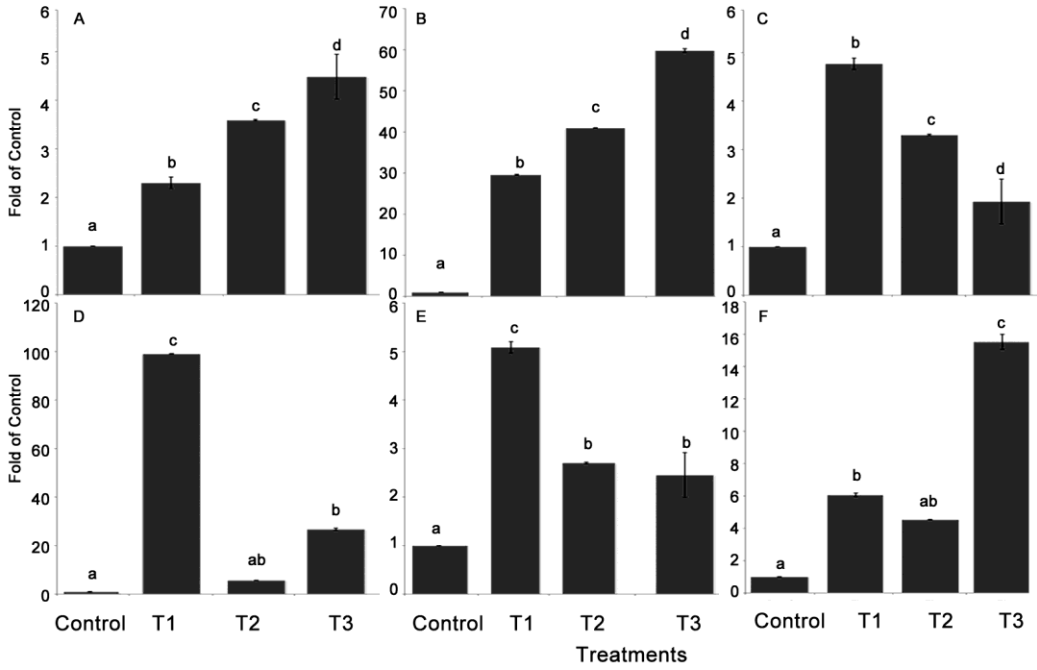

Fig. 2 — Immune related genes expression after dietary administration of BLAL extract. (A) *HSP70* gene expression in kidney; (B) *HSP7* gene expression in liver; (C) *IL-1β* gene expression in kidney; (D) *IL-1β* gene expression in liver; (E) *TNF-α* gene expression in kidne and (F) *TNF-α* gene expression in liver. [Values are presented as mean ± SE. Significant differences (P <0.05) are indicated by differe superscript (a, b, c and d)]

temperature, pH, extraction time, sample particle size, method of extraction and other interfering constituents[27].

Alkaloids are one of the most competent and therapeutically significant compounds known for their potential analgesic, bactericidal, antiviral and anticancer attributes[27,28]. In the present study, the presence of alkaloids was observed in all the extracts; however, BSAQ extract showed maximum alkaloid percentage (27.27%) among other extracts. Similarly, Akinsanya *et al.*[28] also reported the alkaloids from several plant extracts such as *Piper guineense*, *Aframomum melegueta*, *Xylopia aethiopica*, *Gongronema latifolium Moringa oleifera*, *Azadirachta indica* and *Garcinia kola.*

Phenolic compounds are considered as one of the major antioxidant agents because of their redox

potential, which imparts significant role in decomposing peroxides, neutralizing free radicals or triplet oxygen and quenching singlet oxygen[29]. Phenols are also known for the attributes like immune-enhancers, anti clotting, antiinflammatory, hormone-modulator, antioxidant, etc.[28]. In the present study, the presence of phenol was reported in all the extracts; however, BSAL extract showed maximum phenolic content of 41.52 mg GAE g^{-1} of extract. Similar kind of results were also observed by Do *et al*.[30] and Vanitha *et al*.[31] where authors extracted the phenolic compounds from *Limnophila aromatic* and bamboo shoot, respectively and reported that, the hydroalcoholic and alcoholic solvents are much superior in extracting the phenols. Later on Akhtar *et al.*[26] also reported phenol while studying the medicinal plant extract. In the present study, the total phenolic content of alcoholic extracts was higher than the aqueous extract which corroborates the findings of Do *et al.*[30] in the *L. aromatic* extract.

Flavonoids are the largest group of naturally occurring phenolic compounds and potent agent with characteristics like antioxidant, metal chelating ability and potential to reduce lipid peroxidation[32]. In the present study, BSAL extract showed significantly (P <0.05) higher level of flavonoids content (144.19 mg QE g^{-1} of the extract) among all extracts. Present study also revealed, the flavonoid content of alcoholic extract was significantly (P <0.05) high as compared to both aqueous extracts. Present finding is corroborated by the finding of Vanitha *et al*.[31] where authors reported high flavonoids content in alcoholic extract of bamboo shoot. Similarly, Akinsanya *et al.*[28] also reported flavonoids from several plant extracts such as *Piper guineense*, *A. melegueta*, *X. aethiopica*, *G. latifolium*, *M. oleifera*, *A. indica* and *G. kola.*

BLAL extract showed significantly (P <0.05) higher level of ascorbic acid content (3.36 mg AE g^{-1} of extract). Similarly, Bhargava *et al.*[33] have also reported ascorbic acid up to 23 mg 100 g^{-1} in bamboo. Similarly, present study also exhibited that the ascorbic acid content of alcoholic extracts was significantly (P <0.05) high compared to aqueous extracts. This may be due to the superior extraction efficacy of hydroalcoholic or alcoholic solvents when compared to the aqueous solvent[25,26].

In the present study, saponin content of alcoholic extracts was significantly (P <0.05) high compared to aqueous extracts. Where, saponin content of BSAL extract was significantly (P <0.05) high (337 mg DE g^{-1} of extract) than the other remaining three extracts. The presence of saponin in extracts corroborates to the findings of Kuddus *et al.*[34] in Muli bamboo and Vanitha *et al.*[31] which may be the result of higher efficiency of alcohol in extracting phyctoconstituents[25,26]. Akinsanya *et al.*[28] also reported the saponin content in several extracts derived from plants *viz*. *Piper guineense*, *A. melegueta*, *X. aethiopica*, *G. latifolium*, *M. oleifera*, *A. indica* and *G. kola.*

In the present study, tannin content of BLAQ extract (3.6 TAE g^{-1} of extract) was significantly (P <0.05) higher than the other three extracts. In fact, tannin content of the aqueous extracts was significantly (P <0.05) higher than that of the alcoholic extracts. BLAL extract showed the maximum content of rotenone (7.46 mg RE g^{-1} of extract) among all the four extracts. The rotenone content of alcoholic extracts was significantly (P <0.05) higher than that of aqueous extracts. Sae-Yun *et al.*[35] also reported similar type of findings where rotenone from *Derris elliptica* and *D. malaccensis* plant root extract ranging from 15 to 46.1% (w/w) depending upon type of solvent and extraction method. Whereas, Zubairi *et al.*[36] reported rotenone content of about 41.75±3.2 mg mL^{-1} in liquid crude extract of *D. elliptica*.

For determination of antioxidant property, three different radical scavenging assays were performed. DPPH produces free radicals and accepts electron or hydrogen to turn into a stable diamagnetic molecule[32]. BLAL extract shows maximum (80.24%) DPPH radical scavenging activity among all extracts, which may be attributed to the higher presence of ascorbic acid and total phenolic content. This result is an agreement with the findings of Vanitha *et al*.[31] where authors reported elevated free radical scavenging response from bamboo ethanolic extract as compared to other extracts. In the current study, DPPH radical scavenging activity of alcoholic content was higher than aqueous extract which also corroborates to the finding of Do *et al.*[30] in the *Limnophila aromatica* extract. The bamboo extract may act as an electron or hydrogen donors to scavenge free radicals and to convert them into more stable products[37].

Among all extracts, BSAQ extract showed significantly (P <0.05) higher level (95.38%) of H_2O_2 radical scavenging activity. This might be the result of antioxidant activities of phenolic compounds and

alkaloid present in the extract as also reported by Goyal *et al.*[38] while working on *Hippophae salicifolia*. Whereas, BLAQ extract showed significantly (P <0.05) higher alkaline DMSO radical scavenging response (83.36%) among all four extracts. Similar results were also observed by Kuddus *et al.*[34] in Muli bamboo and Vanitha *et al.*[31] in *Bambusa arundinacae*. The antioxidant properties of extracts is cumulative produce of all the phytochemicals present in it, therefore, it is difficult to evaluate the antioxidant potential based on any individual active component neither possible to evaluate through single type of radical scavenging assay[39].

The antimicrobial activity of the extracts could be attributed to different phytochemicals present therein which possibly are responsible for disabling microbial movements, cell membrane transport proteins and enzymes. For the assessment of antimicrobial activity of extracts, agar-well diffusion test was performed. Against fungi, maximum zone of inhibition (ZOI) was exhibited by BSAQ extract with 15.34 mm of clear zone against *S. parasitica*. Whereas against *E. coli*, BSAL extract exhibited the maximum ZOI of 9.64 mm which may be due to the maximum content of flavonoid and phenolic content in shoot extracts. Similar kinds of results were also observed by Kuddus *et al.*[34] in Muli bamboo and Vanitha *et al.* in *B. arundinacae*[31]. Various phytochemicals present in the plant extracts *i.e.*, pyrolysates, flavonoids, dimethoxy-p-benzoquinone, chitin-binding peptide, phyllostachys, stigmastreol, dihydribrassicasterol, tricin, taxifolin, saponin, *etc.* may correspond to the antimicrobial attributes of the extract[40].

Haemolytic study helps in understanding the toxicity effect of extract on the cellular level. The membrane stability of RBCs is a good indicator of the cytotoxic effect of various phytochemicals[42]. In the present study, all the extracts were reported to cause haemolysis; however, the lysis pattern was least for BLAQ extract (2 HU) on chicken blood. Saponin content in extract may be responsible for haemolysis due to the presence of lipid soluble moieties (sapogenin) which allow saponin to lyse RBCs membrane[43]. Other phytochemicals present in the extracts such as rotenone, tannin, etc. may also be responsible for toxic response. The present finding is corroborated by the observation of Debbarma[41] who reported haemolysis of chicken blood up to 9 HU for the extract of *Gliricidia sepium.*

Scoring system (Table 6) was adopted to select the best extract among all four extracts for further *in vivo* study. In the present study, among all the extracts BLAL extract was found to be the best with a cumulative score of 45 out of 64.

For the acute toxicity test, *L. rohita* were fed with purified diets containing graded level of BLAL extract at 2.5, 5, 10 and 20 g kg^{-1} body wt. However, during the experimental trial, fingerlings were stressed (behavioural abnormalities) for some period after feeding maximum dose however, they recovered soon. Similar stressful condition was also observed by Akinsanya *et al.*[28] after feeding multiple extracts to *Clarias geripinus*, which may be due to the presence of certain saponin, rotenone, tannin and alkaloid content in the extract as they known for their detrimental effects. In the present study no mortality was recorded up to maximum dose of 20 g kg^{-1} body weight, which can be regarded as virtually non-toxic with nominal effect. This non-toxic nature of BLAL extract corroborates by the results of Lu *et al.*[42], who found out that, *Phyllostachys nigra* leaf extract up to 4.3 g kg^{-1} body weight for 90 days is nontoxic in rat.

Immune related gene expression analyses was carried out to know the immunological responses caused by BLAL extract incorporated diet at the molecular level, because there are several reports which suggest that many of the bioactive compounds of herbal origin act as immunobooster[43]. The molecular response confirms the results of cellular immune modulation indicating the added advantage of studying the expression profile of immune genes. The two major immunocompetent organs (liver and head kidney) of rohu were considered in the present study for the transcriptional analyses. One heat shock protein (*HSP70*) and two pro-inflammatory cytokine/ chemokines genes (*IL-1β* and *TNF-α*) were chosen for the analyses after feeding with graded level of BLAL extract incorporated diet.

In recent findings of Eden *et al.*[44] reported that HSPs enables long lasting protective immune response and also related to pro-inflammatory damage associated molecular patterns. They also reported that the HSP is responsible for inducing T cells for the production of IL-10[44]. HSP is known for its potent anticancer immune response, facilitated by the innate and adaptive immune system[44]. In the present study, the expression of *HSP70* significantly (P <0.05) increased in both liver and head kidney with the

increasing concentration of BLAL extract in the feed. The elevated expression level of *HSP70* may be because of the phytoconstituents present in the BLAL extract which causes activation of immune cells. Similar kind of results was also observed by Kaleo *et al.*[45] after feeding *Moringa oleifera* leaf extract to *Macrobrachium rosenbergii*. Liu e*t al.*[46] also observed *HSP70* expression in *Megalobrama amblycephala* after feeding them with *Rheum officinale* Bail Extract.

The *IL-1β* imparts role in microbial invasion and can rouse immune response by triggering cytokine expression to stimulate macrophages, lymphocytes and NK cells production[47]. In the present study, *IL-1β* expression was significantly (P <0.05) higher in BLAL extract fed group than control group. However, with the increasing concentration of extract in diet, there is a decreasing pattern in *IL-1β* expression in both liver and head kidney which corresponds to lower dose of extract in diet as better immune enhancing response than elevated dose. Similar result was also observed by Anbazahan *et al.*[48] while working on the effect of carotenoids supplemented diet in *Cyprinus carpio*. The up-regulation of *IL-1β* indicates that there may be some influence of BLAL extract in activating different components of the immune system. Kumar *et al.*[6] conducted an experiment on BALB (Bagg Albino -inbred research mouse strain) where they fed them with *Phyllostachys bambusoides* extract and studied the immune gene expression (*IL-4* and *IL-2*) and found out that there was significant (P <0.05) up-regulation as compared to the control.

TNF-α is one of the major cytokines in the fish immune system, which plays an important role in the immune system of fish. When macrophages interact with foreign invader many types of immune signalling proteins are released. Among the cell signalling proteins, *TNF-α* plays a substantial role in developing cytokine network *e.g.* NK cell and T cell activation[49]. In the present study, *TNF-α* expression in both the tissues was significantly (P <0.05) higher than control where the maximum expression of *TNF-α* was observed at the lowest dose (0.01%) for kidney however, on the contrary liver tissues exhibit maximum expression at maximum dose (1%) of extract. Similar kind of study was also conducted by Yousefi *et al.*[50] and found significant (P <0.05) upregulation of *TNF-α* expression as compared to control. The present immune gene expression analyses revealed the immunomodulatory potential of BLAL extract. Because, with the increasing concentration of extract in the feed, the expression of pro-inflammatory cytokines (*IL-1β* and *TNF-α*) were downregulated significantly in both the tissues except *TNF-α* expression in liver. Additionally, expression of *HSP70* was increased significantly with the increasing levels of dietary BLAL extract.

Conclusion

Present holistic assessment revealed BLAL extract as a significant source phytochemicals (alkaloid, phenol, flavonoids, ascorbic acid, saponin, rotenone and tannin) with superior antimicrobial, antioxidant attributes with no toxic effect on *L. rohita* up to 20 g kg^{-1} body weight. Immune related genes (*HSP70*, *IL-1β* and *TNF-α*) expression analyses revealed the immunomodulatory potential as there was significant expression up on exposure to BLAL extract incorporated diet (control- 0.0%, T1:0.01%, T2:0.1% and T3:1% extract kg^{-1}) in rohu. However, for any field level application further comprehensive studies are required.

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Conflict of interest

Authors declare no competing interests.

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