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Synergistic Effects of Phytogenic Blends on Growth Performance, Nutrient Utilization, Digestive Enzyme Activity and Haemato-immunological Response of Nile Tilapia (*Oreochromis niloticus*) in Natural Pond Ecosystem

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ABSTRACT

Present experiment was carried out during July to October, 2023 at the pond facilities of the Faculty of Fishery Sciences of West Bengal University of Animal and Fishery Sciences, Kolkata, India to evaluate the efficacy of two phytochemical formulations in Nile tilapia fingerlings. Medicinal herbs and their extracts were increasingly favoured in aquaculture due to their immunostimulatory, anti-inflammatory, and growth-promoting properties. A 90-day pond trial was conducted using a completely randomized design with three dietary groups in triplicate. In control there was no additive. In first treatment group a mixture of garlic, methi, and eucalyptus leaf and in second treatment group a blend of thyme, eucalyptus, and clove essential oils were included at 0.5 g kg^{-1}. Assessments included growth performance, feed utilisation, digestive and metabolic enzyme activities, haemato-immunological assays, and serum biomarkers. Results indicated that these phytogenic blends significantly influenced growth trajectories. The treatment group 1 exhibited superior body weight gain, nutrient utilization, and respiratory burst modulation, suggesting a synergistic effect of the herbal combination. Body weight gain (g), specific growth rate and feed conversion ratio values in group 1 were 37.23, 1.68 and 0.75 in comparison to group 2 showing the values 25.41, 1.40 and 1.06, respectively. These findings demonstrated that a dietary phytochemical formulation of garlic, methi, and eucalyptus positively influenced physiological robustness and supported sustainable fish production.

KEYWORDS: Phytochemicals, garlic, methi, eucalyptus, thyme, feed utilization, tilapia

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1. INTRODUCTION

The global demand for sustainable protein has established aquaculture as a vital pillar of international food security, with Nile tilapia (*Oreochromis niloticus*)-frequently termed the 'aquatic chicken'-emerging as a primary species for poverty alleviation and hunger eradication (Anonymous, 2024; Tacon, 2020; El-Sayed and Fitzsimmons, 2023). However, the drive for intensive farming has introduced a productivity-stress paradox, where elevated stocking densities and environmental fluctuations heighten disease risks and financial volatility (Bahi et al., 2017). Historically, the industry has relied heavily on synthetic antibiotics and chemotherapeutants to mitigate these biological pressures. Recent projections indicate that global antibiotic consumption in aquaculture will exceed 13,600 tons by 2030 (Schar et al., 2020). This reliance is ecologically problematic, as up to 80% of these substances enter the aquatic environment unmetabolised, driving the proliferation of antimicrobial resistance (AMR) and threatening human health via persistent tissue residues (Brown et al., 2021). Consequently, research has pivoted toward eco-friendly phytogenic feed additives (PFAs) as viable alternatives. Bioactive compounds in garlic, fenugreek, and various essential oils-such as thyme, eucalyptus, and clove-contain high concentrations of polyphenols and terpenoids, including carvacrol and eugenol, which provide potent antioxidant and immunostimulatory benefits (Ahmadifar et al., 2021; Anastasiou et al., 2020; Dawood et al., 2021). Garlic (*Allium sativum*) contains S-allylcysteine sulfoxide, an odourless sulfur compound that stimulates hypoglycaemic activity while enhancing antioxidant status and immunological markers (Martins et al., 2004; Yılmaz and Ergun, 2012). Investigations in Sobaity sea bream and other species confirm that garlic inclusion significantly improves growth performance and non-specific immunity (Jahanjoo et al., 2018). Similarly, fenugreek (*Trigonella foenum-graecum*) is rich in nitrogenous compounds and polyphenols, exhibiting antidiabetic, anti-inflammatory, and immunomodulatory properties (Mukthamba and Srinivasan, 2016; Olaiya and Soetan, 2014). Studies on gilthead seabream and common carp confirm that fenugreek, administered alone or alongside probiotics, improves growth and innate immunity (Bahi et al., 2017; Roohi et al., 2017; Mehboob et al., 2017). Eucalyptus (*Eucalyptus globulus*), rich in the monoterpenoid eucalyptol, provides robust antimicrobial support (Luis et al., 2016; Aprotosoaie et al., 2019), with supplementation in *O. niloticus* diets leading to improved feed efficiency and haematological markers (Nurudeen et al., 2022). Clove oil, primarily composed of eugenol, is widely accepted as a safe anaesthetic and bactericidal agent against pathogens such as *Salmonella* and *Escherichia* (Kildea et al., 2004; Dorman and Deans, 2000). Furthermore, the FDA-authorised use of clove oil as a food additive underscores its safety profile (Anderson et al., 1997). Its hydroxyl groups facilitate significant antioxidant activity and inhibit cytokine production, indicating a strong anti-inflammatory effect (Bachiega et al., 2012; Gill and Holley, 2006). Clove oil supplementation has been shown to improve phagocytic and respiratory burst activities in *O. niloticus* following pathogen challenges (Abdelkhalek et al., 2020). Thyme (*Thymus vulgaris*) has likewise been demonstrated to enhance growth and immune parameters in rainbow trout and carp (Zargar et al., 2019; Ghafarifarsani et al., 2022).

Recent trends emphasize multi-component formulations to leverage the "entourage effect." Bababaalian et al. (2020) demonstrated that a blend of thyme, oregano, and eucalyptus oils improved hepatic function and oxidative stability in rainbow trout. Similarly, a mixture of six herbs, including fenugreek and thyme, enhanced growth and feed utilisation in monosex tilapia (Tonsy et al., 2011). Despite these advancements, a significant research gap persists; most phytochemical evaluations are conducted in sterile laboratory tanks, which lack the complex microbial diversity and variable chemistry of natural ponds-the systems most commonly utilised by commercial farmers (Bahi et al., 2017). Furthermore, the synergistic potential of specific multi-herb blends in pond settings remains largely unexplored. This study was aimed to evaluate two distinct phytochemical formulations on the growth, metabolic enzyme activity, and immunological responses of *O. niloticus* within a natural pond ecosystem, providing an ecologically valid assessment of sustainable aquaculture practices.

2. MATERIALS AND METHODS

2.1. Experimental site

Present experiment was carried out during July to October, 2023 at the pond facilities of the Faculty of Fishery Sciences of West Bengal University of Animal and Fishery Sciences, Chakgaria, Kolkata, India (Lat.22.4796 °N; Long. 88.4012 °E) for a period of 90 days. Earthen ponds of the farm complex were selected for the study and nine rectangular hapa were made using bamboo structures with small mesh size net.

2.2. Experimental design

A completely randomized design (CRD) was followed for the experiment with three replicates for each three groups (3×3). The pond was prepared following the usual pre-stocking procedures of a fish culture, which included drying the pond followed by excavation of muddy layer. Quick lime (CaO) was applied at 1.5 kg ft^{-2} in order to buffer the pH and also to eradicate parasites and pathogens. Later water was filled to maintain the level of at least 4 ft. After checking the water quality parameters and plankton growth in the pond total 720 juvenile Nile tilapia (*Oreochromis niloticus*) which were already acclimatized, were randomly distributed; 80 fish in each replicate compartment. Initially fish body weights were taken at the time of allocation and the mean body weight was maintained over the all replicates.

2.3. Formulation of experimental diets

Three iso-nitrogenous and iso-lipidic experimental diets were formulated, the details of which were provided in Table 1. The control group (CON) received a basal diet devoid of phytochemical supplementation. The first treatment group (TR1) was administered a diet enriched with a phytochemical formulation (PCF-1) comprising garlic, methi, and eucalyptus leaf powder. The second treatment group (TR2) received the same basal diet supplemented with a second formulation (PCF-2), consisting of clove, eucalyptus, and thyme essential oils. Both PCF-1 and PCF-2 were included at a concentration of 0.5 g kg^{-1}. These formulations were procured from Ayurvet Limited (Himachal Pradesh, India), where they were blended and supplied under the trade names Nbiotic and AV/EIF/19, respectively. All feed ingredients were of high quality, sourced from the Kalyani Feed Milling Plant (West Bengal Livestock Development Corporation Limited, Nadia, West Bengal). The preparation process involved grinding, sieving, and weighing the raw materials according to the formulation. The mixture was then steamed for 30 minutes using a pressure cooker to ensure starch gelatinisation. To preserve their bioactive properties, all heat-sensitive additives-including phytochemicals, vitamins, and mineral premixes-were homogeneously incorporated only after the mixture had cooled. The resulting dough was processed through a pelletizer to create uniform pellets, which were spread on metal trays and air-dried. The dried pellets were subsequently crumbled to a size compatible with the mouth opening of the experimental fish and stored under optimal conditions. For quality control, triplicate samples of each diet were ground into a fine powder for proximate analysis. The chemical composition of the diets was determined following Anonymous (2010) protocols, as summarised in Table 1.

2.4. Feeding and maintenance of experimental fish

The experimental fish were fed to satiation level thrice daily into equal proportion during 09:00 a.m. in the morning; 03:00 p.m. in the afternoon and 07: 00 p.m. in the evening, respectively throughout the experimental period of 90 days. Feeding was done at 3% of the biomass and to adjust the level of feeding, the weight of the fish was assessed every 15 days intervals.

2.5. Water quality analysis

Water quality parameters were assessed between 09:00 a.m. and 11:00 a.m. on weekly basis. The pH and temperature were measured using pH meter and thermometer. Dissolved oxygen, hardness, ammonia, nitrite and nitrate were measured using test kits (Life Sonic Innovations Pvt. Ltd., India; Nice Chemicals Pvt. Ltd, India).

Table 1: Formulation and proximate composition of experimental diets supplemented with different phytochemical formulations

Ingredients (g kg^{-1})	Experimental diets		
	CON[1]	TR1[2]	TR2[3]
Fish meal (FM)	170	170	170
De-oiled rice bran (DORB)	250	250	250
Mustard oil cake (MOC)	100	100	100
Soya bean meal	280	280	280
Corn meal	80	80	80
Wheat flour	100	99.5	99.5
Soya oil	10	10	10
[4]Vitamin and mineral premix	10	10	10
PCF-1	-	0.5	-
PCF-2	-	-	0.5
Total (g)	1000	1000	1000
Chemical compositions (% dry matter basis, Mean±SD, n= 3)			
Crude protein (CP%)	30.3±0.43	30.12±0.66	30±0.25
Crude lipid (CL%)	9.96±0.66	10±0.8	10.08±0.23
Total ash (TA%)	7.5±0.15	7.58±0.35	7.66±0.3
Gross energy (Kcal kg^{-1})	4123±1.15	4125±1.4	4126±1.37

[1]CON: Control feed; [2]TR1: Control feed with PCF-1 supplementation (prepared using garlic, methi and eucalyptus leaf powder; Nbiotic); [3]TR2: Control feed with PCF-2 supplementation (prepared using clove oil, eucalyptus oil and thyme oil; AV/EIF/19). Both the formulations were produced by Zenex Animal Health India Private Limited and supplied for the present study; [4]Composition of the Vitamin and mineral mixture (quantity kg^{-1}): Vitamin A, 26,00,000 IU; Vitamin D3, 8,00,000 IU; Vitamin B1, 6000 mg; Vitamin E, 100 g; Vitamin K3, 6000 mg; Vitamin B2, 16000 mg; Vitamin B6, 10000 mg; Vitamin B12, 4.4 mg; Pantothenic acid, 3000 mg; Niacin, 10 g; Biotin, 300 mg; Folic acid 4000 mg; Choline chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 mg; Selenium, 125 mg

2.6. Growth performance and feed utilization

Fish were weighed from each replicate at 15 days interval throughout the 90 days of feeding trial following 6 hrs of fasting using a digital weighing balance. The growth and feed utilization parameters like live weight gain, body weight gain percentage, specific growth rate percentage, feed conversion ratio and survival percentage, were calculated by using standard formulas.

WG (%)={(Final wet weight (g)-initial wet weight (g)/ Initial wet weight (g)}×100

SGR (%/day)={ln (final wet weight) -ln (initial wet weight))/Experimental period (days)}×100

FCR=Feed intake (g)/Body weight gain (g)

Survival (%)=(Number of fish harvested/Number of fish stocked)×100

2.7. Sample collection

Upon conclusion of the 90-day feeding trial, 10 fish were randomly harvested from each replicate and anaesthetized using clove oil at a concentration of 50 µl l^{-1}. Blood was drawn from the caudal vein using sterile syringes. For haematological analysis, a portion of the blood was transferred to vials coated with 2.7% EDTA as an anticoagulant. To obtain serum, the remaining blood was collected in additive-free vials and allowed to clot at 4°C for one hr. The samples were then centrifuged at 5000 rpm for 15 min, and the resulting serum was immediately transferred to Eppendorf tubes and stored at -20°C for subsequent biochemical analysis. Following blood collection, the fish were dissected to excise the intestine and liver upon an ice pack. These tissues were promptly homogenised to prepare a 5% (w/v) tissue homogenate using a chilled 0.25 M sucrose solution. The homogenates were centrifuged at 5000 rpm for 10 min at 4°C, after which the supernatant was collected in 2 ml Eppendorf tubes and preserved in a deep freezer at -20°C. For the various enzymatic assays, the samples were diluted accordingly to meet specific analytical requirements.

2.8. Enzyme assays

2.8.1. Digestive enzyme activities

Intestinal protease activity was assayed following casein digestion method described by (Drapeau, 1976) and one unit of protease activity was expressed as millimole of tyrosine released min^{-1} mg^{-1} protein. Using the di-nitrosalicylic acid (DNS) method, the amylase activity was calculated as the reducing sugars generated as a result of the action of gluco-amylase and beta-amylase on carbohydrates by following (Rick and Stegbauer, 1974). Amylase activity was expressed in moles of maltose liberated from starch per minute at 37°C. The lipase activity was determined using (Cherry and Crandall, 1932) titrimetric method, based on the measurement of fatty acids produced by the enzymatic hydrolysis of triglycerides contained in an olive oil stabilized emulsion. Lipase activity was expressed in unit hr^{-1} mg^{-1} protein.

2.8.2. Aminotransferase enzyme activities

Aspartate aminotransferase (AST) activity was assayed in tissue homogenates of liver as described by (King and Wootton, 1964). The specific AST activity expressed as nanomoles oxaloacetate released mg^{-1} protein min^{-1} at 37°C. Alanine aminotransferase (ALT) was determined using the same method except the substrate consisted 0.2 M D, L-alanine instead of D, L-aspartic acid. The enzyme activity was expressed as nanomole pyruvate formed/min/ mg protein at 37°C.

2.8.3. Phosphatases activities

Intestinal alkaline phosphatase (ALP) activity was assayed by the method described by (Akinpelu et al., 2012). Similarly, acid phosphatase (ACP) activity was determinate except the buffer, which was 0.2 M sodium acetate– acetic acid mixture with pH 5 in latter. Phosphatases activities were expressed in micromoles of paranitrophenol (PNP) released min^{-1} mg^{-1} protein.

2.9. Haematological profile

The haemoglobin level was estimated by the acid-haematin method with Sahli's haemoglobinometer (Hesser, 1960). Total erythrocyte count (TEC) and total leukocyte count (TLC) were done by using hayem's fluid and turk's WBC dilution fluid respectively in the Neubauer's counting chamber and calculated using the formulae described by (Das et al., 2022).

2.10. Nitro blue tetrazolium (nbt) assay

The respiratory burst activity of the leucocytes was assessed using the method described by (Devi and Kamilya, 2019) as adapted from (Anderson and Siwicki, 1995). The optical density of the turquoise blue coloured solution formed after adding potassium hydroxide (KOH) and dimethyl sulphoxide (DMSO) in the wells fixed with nitro blue tetrazolium (NBT) was then read in ELISA reader.

2.11. Serum biochemicals

Serum glucose was estimated following the methods developed by (Bartonkova et al., 2017). Commercial kit (Innolin TM Total Protein Plus, Merck, India) was used to determined serum total protein (TP), albumin and globulin was calculated by subtracting the albumin content from total protein content. Albumin to globulin ratio (A:G) was calculated by dividing the albumin with globulin.

2.12. Statistical analysis

All experimental data were expressed as a mean±standard deviation (SD). One-way analysis of variance (ANOVA) carried out using the IBM-SPSS version: 22.0, for comparison of means at suitable probability level along with Duncun post-hoc multiple comparison, considering the probability level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Water quality and growth performance analysis

Physico-chemical parameters of water in experimental groups such as temperature (°C), dissolved oxygen (mg l^{-1}), pH, total hardness (mg l^{-1}) and ammonia (mg l^{-1}) are illustrated in Table 2. Utilizing herbal preparations to enhance aquaculture performance is generally more cost-effective and environment friendly than conventional methods. In the present study, water quality parameters remained largely unaffected by the treatments. Feeding of phytochemical formulations showed a significant effect on growth performances and nutrient utilization among the treatment groups (Table 2). However, significantly ($p<$ 0.05) higher body weight gain was attained by the fishes of TR1 group. Phyto-additives supplemented groups exhibited significantly ($p<0.05$) better feed utilization than control. The phytochemical blends used in this trial improved the overall performance of Nile tilapia within a natural pond environment. While the average final body weight of the experimental fish increased over time across all groups, the magnitude of this growth varied significantly depending on the supplementation level. Generally, phyto-additives included at a level of 0.5 g kg^{-1} promoted higher body weights throughout the study period (Figure 1). Furthermore, the selected herbs had a significant impact on growth performance and nutrient utilization among treatment groups (Table 2). Specifically, the TR1 group attained a significantly higher body weight gain ($p<0.05$). Notably, both phyto-additive groups exhibited significantly better feed utilization ($p<0.05$) compared to

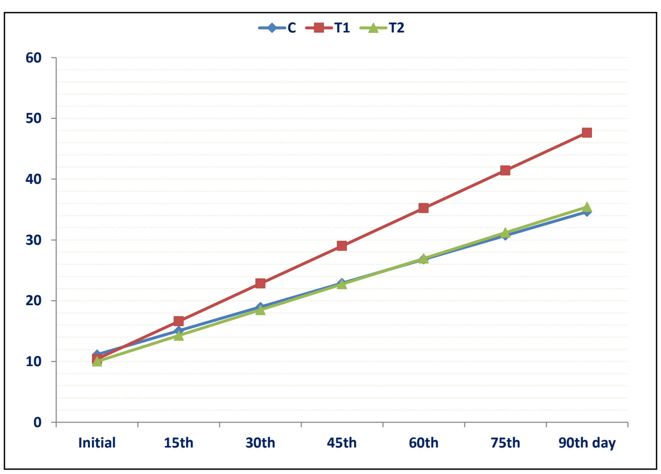

Figure 1: Trends of live body weight attained among test groups (Day 1–Day 90)

Table 2: Water quality parameters in experimental pond and growth performances of experimental fish

	[1]CON	[2]TR1	[3]TR2	p value
Water quality parameters				
Temperature (°C)	28.83± 1.23	28.66± 1.17	29.06± 1.20	0.921
Dissolved oxygen (mg l^{-1})	6.00± 0.26[c]	7.16± 0.70[a]	7.03± 0.35[b]	0.046
pH	6.9± 0.26	7.2± 0.36	7.2± 0.25	0.339
Hardness (mg l^{-1})	256.33± 3.21	256.33± 5.13	255.66± 4.50	0.977
Ammonia (mg l^{-1})	0.08± 0.01[a]	0.04± 0.005[b]	0.05± 0.005[b]	0.007
Growth performances				
Initial body wt (g)	11.13± 0.78	10.41± 0.92	10.02± 0.55	0.333
Final body wt (g)	34.65± 1.68[b]	47.64± 1.10[a]	35.43± 0.69[b]	0.001
Body wt gain, BWG (g)	23.52± 0.98[c]	37.23± 0.18[a]	25.41± 0.22[b]	0.001
BWG (%)	211.55± 9.09[c]	359.50± 31.48[a]	253.90± 13.18[b]	0.001
Specific growth rate (SGR)	1.261± 0.032[c]	1.689± 0.075[a]	1.403± 0.041[b]	0.001
Feed conversion ratio (FCR)	1.27± 0.054[a]	0.75± 0.003[c]	1.06± 0.009[b]	0.001

All values were expressed as Mean±SD. Mean values in the same row with different superscript differ significantly ($p<0.05$); [1]CON: Control feed; [2]TR1: Control feed with PCF-1 supplementation (prepared using garlic, methi and eucalyptus leaf powder, Nbiotic); [3]TR2: Control feed with PCF-2 supplementation (prepared using clove oil, eucalyptus oil and thyme oil; AV/EIF/19)

the control. These results align with Zaki et al. (2012), who observed that adding fenugreek (methi), eucalyptus, or thyme to diets positively influenced weight gain and feed efficiency in Nile tilapia (*O. niloticus*). Similarly, Jimoh et al. (2024) and Diab et al. (2023) reported that fenugreek seed supplementation significantly improved growth. Our findings further support previous research demonstrating the positive role of phytochemicals-such as garlic, methi, and eucalyptus powders or essential oils of clove, eucalyptus, and thyme-in promoting growth and feed utilization across various fish species (Bahi et al., 2017; Jahanjoo et al., 2018; Moustafa et al., 2020; Nurudeen et al., 2022; Tonsy et al., 2011; Zargar et al., 2019). Conversely, some studies have suggested that herbal combinations may have antagonistic effects; for instance, Fawole et al. (2020) and Chowdhury et al. (2021) reported negative impacts on growth when using certain phyto-mixtures. However, the results of the current experiment indicate no such antagonism; instead, the phytochemicals demonstrated a synergistic effect, successfully improving both growth performance and feed efficiency.

3.2. Digestive and metabolic enzyme activities

3.2.1. Digestive enzymes

Significantly higher (p<0.05) activities of protease and amylase were recorded in the phyto-additives supplemented groups compared to the control group (Table 3). TR1 group exhibited significantly higher (p<0.05) digestive enzyme activity than others. Among the three digestive enzymes evaluated, the phytochemical-supplemented groups exhibited significantly higher (p<0.05) protease and amylase activities compared to the control group (Table 3). Specifically, the TR1 group showed significantly higher (p<0.05) digestive enzyme activity than all other treatments. Digestive enzymes such as protease, lipase, and amylase provided critical insights into nutrient utilization efficiency; they directly influenced growth performance by enhancing feed absorption (Lemieux et al., 1999). The comprehensive effect of various herbs and their essential oils on modulating digestive enzymes has been well-documented across several species, including poultry (Jang et al., 2007), ruminants (Parkunan et al., 2024), and fish (Magouz et al., 2021). These additives accelerated feed utilization and improved the overall well-being of livestock in production systems. For instance, eucalyptus oil administered at a 5% level yielded the best performance in tilapia, likely due to improved digestion and absorption triggered by stimulated protease release in the intestinal tract (Nurudeen et al., 2022). Similar improvements have also been reported in groupers (Putra et al., 2013).

Table 3: Digestive and metabolic enzyme activities of experimental fish

	[1]CON	[2]TR1	[3]TR2	p value
Digestive enzymes activities				
Protease*	0.353± 0.015^{b}	0.400± 0.026^{a}	0.380± 0.02^{ab}	0.042
Amylase*	37.65± 1.10^{c}	41.15± 0.77^{a}	40.0± 1.05^{ab}	0.009
Lipase*	0.37± 0.03	0.35± 0.04	0.37± 0.02	0.379
Metabolic enzymes activities				
AST**	22.04± 1.86	19.68± 0.79	18.99± 3.19	0.278
ALT**	10.02± 1.49^{a}	7.85± 1.79^{b}	8.26± 2.4^{b}	0.031
ACP**	0.449± 0.001^{c}	0.628± 0.002^{a}	0.503± 0.001^{b}	0.001
ALP**	0.685± 0.005^{c}	1.122± 0.003^{a}	1.033± 0.03^{b}	0.001

All values were expressed as Mean±SD. Mean values in the same row with different superscript differ significantly (p<0.05); [1]CON: Control feed; [2]TR1: Control feed with PCF-1 supplementation (prepared using garlic, methi and eucalyptus leaf powder; Nbiotic); [3]TR2: Control feed with PCF-2 supplementation (prepared using clove oil, eucalyptus oil and thyme oil; AV/EIF/19). *Protease activity is expressed in millimole of tyrosine released min^{-1} mg^{-1} protein; Amylase activity is expressed in micromole maltose released min^{-1} mg^{-1} protein; Lipase activity is expressed in units hr^{-1} mg^{-1} protein; v**AST (Aspartate aminotransferase) specific activities expressed as nanomoles oxaloacetate released mg^{-1} protein min^{-1} at 37°C; ALT (Alanine aminotransferase) specific activities expressed as nanomoles of sodium pyruvate released mg^{-1} of protein min^{-1} at 37°C; ACP (Acid phosphatase) activity is expressed as micromoles of paranitrophenol (PNP) released min^{-1} mg^{-1} protein; ALP (Alkaline phosphatase) activity is expressed as micromoles of paranitrophenol (PNP) released min^{-1} mg^{-1} protein

3.2.2. Activity of aminotransferase enzymes

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities in the liver of *O. niloticus* were depicted on Table 3. A significantly lower (p<0.05) ALT values exhibited by supplemented diet groups whereas liver AST value showed no significant (p<0.05) differences. In addition to digestive enzymes, the study monitored aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These enzymes played a vital role in redistributing amino-nitrogen among amino acids. AST catalyzed the interconversion of aspartate and alpha-ketoglutarate into oxaloacetate and glutamate while ALT facilitated the transfer of an amino group from alanine to α-ketoglutarate, converting glutamic acid into pyruvate. The resulting pyruvate and oxaloacetate were essential precursors for the synthesis of non-essential amino acids, such as alanine, asparagine, and glutamine (Silva and Anderson, 1994). In the present study, phytobiotic supplementation led to a reduction in alanine aminotransferase (ALT) levels, although aspartate aminotransferase (AST) levels remained statistically insignificant (p<0.05) among the treatment groups. These results were partially supported by (Zare et al., 2021), who reported that garlic inclusion resulted in non-significant (p<0.05) aminotransferase values in perch. Similarly, (Saleh et al., 2018) observed AST and ALT values within the normal physiological range when using a phytogenic mixture of spearmint, thyme, yucca, pepper, black cumin, onion, and garlic. Conversely, (Imbabi, 2024) demonstrated that including garlic in the diet resulted in significantly lower (p<0.05) ALT and AST levels compared to a diet supplemented with thyme.

3.2.3. Activity of phosphatase enzymes

Alkaline phosphatase (ALP) and acid phosphatase (ACP) of intestinal homogenates of *O. niloticus* depicted significantly higher values after phyto-additives supplementation (Table 4). In the current study, highest phosphatase activity found in TR_2 diet fed group. According to (Blasco et al., 1993), alkaline phosphatase (ALP) and acid phosphatase (ACP) were membrane-bound enzymes that catalyzed the hydrolysis of various phosphate-containing compounds at alkaline and acidic pH levels, respectively. In higher animals, ALP was essential for bone formation, glycogen metabolism, and the transport of metabolites across cell membranes (Molina et al., 2005). ACP was released by lysosomes upon their fusion with secondary lysosomes or digestive vacuoles, initiating the intracellular chemical reactions required for food degradation (Akinpelu et al., 2012). A study by (Singh et al., 2013) revealed that green tea leaf extracts (administered at 4 mg 100 g^{-1} body weight) caused a significant increase in ALP and ACP activity in cadmium-stressed rats. In the current trial, phosphatase enzyme activity was significantly higher in the phytochemical-supplemented diets, with the TR1 group exhibiting the highest values. Consistent with these findings, (Elkomy and Elsaid, 2015) reported that a combination of herbs (sage, rosemary, and thyme) increased both ALP and ACP activity in albino rats.

Table 4: Haemato and serum biochemical parameters of experimental fish

	[1]CON	[2]TR1	[3]TR2	p value
Haematological parameters				
Haemoglobin (g dl^{-1})	5.89± 0.14^{b}	8.51± 0.57^{a}	6.93± 1.12b	0.008
Total erythrocyte count (10^6 cell mm^{-3})	1.55± 0.05^{b}	3.01± 0.21^{a}	2.26± 0.49a	0.003
Total leukocytes count (10^3 cell mm^{-1})	23.82± 0.45^{c}	24.77± 0.55^{b}	26.03± 0.29a	0.001
Nitroblue tetrazolium activity (OD at 540 nm)	0.68± 0.1^{b}	1.09± 0.11^{a}	0.81± 0.06b	0.003
Serum biochemical parameters				
Glucose (mg dl^{-1})	74.95± 4.93^{b}	85.27± 4.53^{a}	82.16± 2.73a	0.010
Total protein (g dl^{-1})	3.57± 0.03	3.50± 0.02	3.42± 0.11	0.144
Albumin (g dl^{-1})	2.41± 0.02^{a}	2.13± 0.01^{b}	2.11± 0.02b	0.001
Globulin (g dl^{-1})	1.16 ± 0.03^{ab}	1.37± 0.04^{a}	1.04± 0.09b	0.001
A:G (Albumin:Globulin)	2.08± 0.07^{ab}	1.54± 0.05^{b}	2.29± 0.23a	0.001

All values were expressed as Mean±SD. Mean values in the same row with different superscript differ significantly (p<0.05); [1]CON: Control feed; [2]TR1: Control feed with PCF-1 supplementation (prepared using garlic, methi and eucalyptus leaf powder; Nbiotic); [3]TR2: Control feed with PCF-2 supplementation (prepared using clove oil, eucalyptus oil and thyme oil; AV/EIF/19)

3.3. Haematological parameters

Hematological indices of *O. nloticus* fed different phyto-additive supplemented diet differ significantly (p<0.05) (Table 4). Different blood parameters such as, Haemoglobin (Hb) and total erythrocyte count (TEC) exhibited higher value in TR_1 group except total leucocyte count (TLC). Along with the metabolic enzyme activities haematological indices exhibited better result in TR1 group. Similarly, previous studies demonstrated garlic (Talpur and Ikhwanuddin, 2012), eucalyptus oil (Sheikhzadeh et al., 2011), methi (Roohi et al., 2017), thyme (Ghafarifarsani et al., 2022) and clove (El-Gindy et al., 2021) exhibited improved haematological profile than control diet group in varied species. Among the phytochemical supplements used in present study, fenugreek and clove were known to have appetite stimulant action, while garlic and thyme was having digestion stimulant action (Cristea et al., 2012) which ultimately along with eucalyptus showed significant performances in terms of digestion, growth, metabolism, haematological and nutrient utilization data.

3.5. Respiratory burst activity

Nitroblue tetrazolium (NBT) activity was significantly (p<0.05) increased in TR_1 supplemented group (T_1). In comparison to control, fish of TR_1 and TR_2 exhibited higher value of respiratory burst activity (Table 4). In the present study, nitroblue tetrazolium (NBT) activity was significantly (p<0.05) increased in TR_1 group. In comparison to control, fish of TR1 and TR_2 exhibited higher value of respiratory burst activity (Table 4). When cells encounter a pathogen, they activated the dormant metabolic pathway known as respiratory burst. Pathogens were eliminated by producing potent oxidizing compounds (ROS) responsible for superoxide anion productions (Dale et al., 2008). The administration of different herbs such as garlic, methi, eucalyptus leaf, thyme oil, clove oil, and eucalyptus oil as a dietary additives has been documented in various fish species, such as 5 g kg^{-1} garlic to juvenile hybrid tilapia (Ndong, 2011), fenugreek seed in gilthead seabream diet at 10% level (Guardiola et al., 2018), *Eucalyptus globolus* at 120 mg kg^{-1} in the diet of common carp (Sheikhzadeh et al., 2011), thyme essential oil at 20 g kg^{-1} in common carp (Ghafarifarsani et al., 2022) and clove at 10 g kg^{-1} in Asian sea bass diet (Talpur and Ikhwanuddin, 2012) increased superoxide anion production (p<0.05). Contrarily, the findings of (Zare et al., 2021) showed the effect of dietary garlic powder has no significant (p<0.05) variation on respiratory burst activity in eurasian perch. Although in the present findings dietary use of herbs in two different combinations exhibited higher NBT values than control group, whereas better improvement occurred in the garlic, methi, and eucalyptus leaf combined diet group. However, a combination of *Thymus vulgaris, Origanum vulgare,* and *Eucalyptus* sp. essential oils supplementation in 56 feeding trials of rainbow trout diet increased the nonspecific immune response (Bababaalian et al., 2020). Supported by (Adeshina et al., 2018) and (Zorriehzahra et al., 2021) who reported supplementation of 15 g kg^{-1} dietary clove to *Clarias gariepinus* and 100 mg kg^{-1} garlic to *Mugil cephalus* enhanced significantly (p<0.05) the respiratory burst activity, respectively. Additionally, (Yılmaz et al., 2016) reported dietary supplementation of herbs increased the NBT level than the non-supplemented group although at higher NBT levels through supplementation of fenugreek and thyme, values did not significantly (p<0.05) differ from each other.

3.6. Serum biochemical responses

In the present study dietary phytobiotic supplementation increased serum glucose level as depicted in Table 4. However, total protein (TP) and albumin (ALB) content decreases with supplementation whereas TR_2 group showed significantly higher (p<0.05) albumin to globulin ratio. The dietary inclusion of phytochemicals significantly influenced several serum biochemical parameters (p<0.05), specifically glucose, albumin, globulin, and the albumin-to-globulin (A:G) ratio; however, total protein levels remained unaffected. These results aligned partially with the findings of (Aljumaah et al., 2020), who reported that while a complex herbal blend (including oregano, eucalyptus, and garlic) did not significantly alter serum total protein, albumin, or globulin, it did lead to a significant reduction in glucose levels (p<0.05). In terms of protein fractions, our observed reduction in serum albumin levels was consistent with the previous study (Jahanjoo et al., 2018), where the inclusion of garlic and thyme in fish diets produced a similar downward trend. Conversely, the immunological profile was enhanced in the fenugreek-supplemented group (TR1), which exhibited higher globulin levels compared to the thyme-oil-supplemented group (TR2). This elevation in globulin-a key indicator of innate immunity-was supported by (Guardiola et al., 2018) and (Emeish and Saad El-Deen, 2016), who noted that fenugreek inclusion stimulates protein synthesis related to the fish's humoral immune response.

4. CONCLUSION

Dietary inclusion of the garlic, methi, and eucalyptus blend (TR_1) exerted superior synergistic effects on Nile tilapia growth, feed utilisation, and metabolism compared to TR_2, the essential oil mixture. TR_1 induced robust immunostimulation, specifically enhancing respiratory burst activity without compromising intestinal enzyme integrity. Consequently, TR_1 is a viable additive for optimising health and production while reducing antibiotic reliance. Future research should evaluate varying dosages and genomic mechanisms to fully elucidate these growth-promoting and immunomodulatory pathways.

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