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Effects of dietary turmeric and clove powder on growth and immune response of the Nile tilapia

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ABSTRACT

A feeding trial was conducted to investigate the effect of dietary incorporation of turmeric powder (TP), *Curcuma longa* and clove bud powder (CBP), *Syzygium aromaticum* on growth, antioxidant, and immune response of Nile tilapia, *Oreochromis niloticus*, as well as its resistance to pathogenic bacteria, *Proteus mirabilis*. Fish (27.56 ± 0.15 g) were randomly allocated into four groups in triplicates. The first (control) group was fed basal without any feed addition. The second and third groups were fed on basal diet enriched with 0.5% TP and 3% CBP, respectively, and the fourth group was fed on a basal diet enriched with a mixture of TP and CBP for 6 weeks. After the 6 weeks of feeding, fish were intraperitoneally injected with *P. mirabilis* and mortalities were recorded up to 14 days. Fish growth parameters were significantly improved in all groups fed TP and/or CBP enriched diets as compared to the control group. The immune response of fish that were fed on dietary TP+CBP mixture followed by TP then CBP before and after bacterial challenge showed significant augmentation in terms of lysozyme activity, nitric oxide, and total protein especially total globulin, as well as the hepatic level of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD) activities and reduced glutathione content (GSH)] over those of the control fish. The fish challenged with *P. mirabilis* showed the highest mortality rate and lowest relative percentage of survival; whereas the highest RPS was recorded in the fish fed TP+CBP mixture and TP (75%). Hence, TP and/or CBP could be used as a dietary additive to improve growth and immune response as well as to protect Nile tilapia against *P. mirabilis* infection.

INTRODUCTION

Globally, Nile tilapia, *Oreochromis niloticus* (L.), is one of the most important freshwater fish species owing to its high nutritional values, rapid growth rate, and tolerance to the bad environment **(Abdel Rahman *et al*., 2019; FAO, 2019)**. However, bacterial disease outbreak continues to occur among cultured fish due to their intensification in farms, causing high mortalities and considerable economic losses **(Austin and Austin, 2007; Ashiru *et al*., 2011)**. Among the bacterial infection that has

been implicated as potential fish pathogens is *Proteus* species **(Daly and Aoki, 2011; Saad EL-Deen, 2013)**.

Proteus mirabilis is an opportunistic, Gram-negative bacterium that belongs to the Enterobacteriaceae family. *P. mirabilis* inhibits the intestine of humans and animals and its presence in fish is a strong indicator of sewage pollution **(Różalski *et al.*, 2012; Drzewiecka, 2016)**. Previously, different *Proteus* spp. induced mortalities in ornamental fish, *Cyprinus carpio koi* **(Kumar *et al.*, 2015)**, Nile tilapia, and red swamp crayfish, *Procambarus clarkii* **(Saad El-Deen, 2013)**. Also, it was reported that *P. mirabilis* induced disease signs including hemorrhages on all body surfaces, histopathological lesions in vital organs, and mortalities in Indian major carp, *Labeo rohita* **(Pattanayak *et al.*, 2018)**.

Generally, using chemotherapeutics in controlling fish bacterial diseases is no longer productive and maintainable owing to their negative impacts on aquatic organisms, the surrounding ecosystem, and human health **(Heuer *et al.*, 2009)**. An alternative strategy should be proposed to be used for combating bacterial diseases and protect fish from these hazards. The plant-based additives are ones of these alternatives **(Reverter *et al.*, 2014; Dada, 2017; Abdel Rahman *et al.*, 2019)**. Medicinal plants are an abundant source of nutrients and antioxidants that can improve fish growth and immunity and consequently, they can be used to control diseases **(Abdel Rahman *et al.*, 2018a and 2019)**.

Turmeric, *Curcuma longa*, is a flowering rhizomatous plant belonging to the medicinal family of Zingiberaceae **(Amalraj *et al.*, 2017)**. It is commonly used as a coloring material, cosmetics, and treatment of many diseases in different countries **(Hatcher *et al.*, 2008; Mahmood *et al.*, 2015)**. Turmeric contains many active components such as curcumin, turmerone, 1,8-cineole, zingiberene, ar-turmerone, and ascorbic acid that have powerful antioxidant activities **(Gounder and Lingamallu, 2012; Dosoky *et al.*, 2019)**. Moreover, it exerts a wide variety of pharmacological activities such as immunomodulatory **(Gupta *et al.*, 2013)**, antibacterial **(Tajbakhsh *et al.*, 2008)**, anti-inflammatory **(Bereswill *et al.*, 2010)**, hepatoprotective, antitoxic, and antimutagenic **(Prasad and Aggarwal, 2011)**. Previously, turmeric powder (TP) or its extracts could boost the growth and immune parameters of Nile tilapia and common carp, *Cyprinus carpio* against invading pathogens **(Elgendy *et al.*, 2016; Abdel-Tawwab and Abbass, 2017; Al-Faragi and Hassan, 2017)**, respectively.

Clove, *Syzygium aromaticum*, is an aromatic medical plant of the family *Myrtaceae*. It is commonly applied as a natural additive in the food industry, antiseptic against infectious diseases, and local anesthetic in dentistry **(Cortés-Rojas *et al.*, 2014)**. Eugenol, eugenyl acetate, carvacrol, tanene, and thymol were detected as major constituents of the clove **(Amelia *et al.*, 2017)**. Clove essential oil has antimicrobial, anti-inflammatory, cytotoxic, anesthetic, and anti-oxidants properties **(Chaieb *et al.*, 2007; Gülçin *et al.*, 2012; Soni and Dahiya, 2014)**. In fish, clove oil could promote the growth of Nile tilapia when added to diets **(Gaber, 2000)**. Also, clove bud powder (CBP) or its extracts was used as an anesthetic instead of chemicals in fish owing to its low costs, simple obtaining, high efficiency, and without any toxicity **(Abdulrahman *et al.*, 2018; Okey *et al.*, 2018)**. Therefore, the current study was carried out to evaluate the role of dietary TP and CBP on growth, antioxidant, and immune status of Nile tilapia as well as its resistance to *P. mirabilis* infection.

MATERIALS AND METHODS

Diets preparation

Both TP and CBP were obtained from a local market in Zagazig city, Egypt. Four experimental diets were prepared by adding TP and/or CBP to the ingredients of the basal diet (32.02% crude protein) at the proportions of 0.0 (control), 0.5 % TP **(Mahmoud *et al*., 2014)**, 3% CBP diet **(Rattanachaikunsopon and Phumkhachorn, 2009)**, and their mixture (0.5 % TP and 3% CBP diet). The ingredients used for the formulation of the basal diet and its chemical analysis were showed in Table 1 according to **NRC (2011)**. The ingredients were mechanically mixed and pelletized using a meat mincer equipped with a 1.5 mm. These pellets were air-dried then were stored at 4 °C in a refrigerator until further use.

Table 1. Proximate chemical composition of the basal diet (%).

Ingredients	%
Yellow corn	30
Soybean meal, 48%	20
Meat meal high fat, 50%	18
Wheat flour	10
Fish meal, 60%	15
Vegetable oil	5.5
Vitamins and minerals mixture [1]	1.5
Total	100
Chemical analysis (%) [2]	
Dry matter	86.02
Crude protein	32.02
Ether extract	9.93
Crude fiber	1.74
Ash	10.13
Nitrogen free extract	35.97
Digestible energy , Kcal/ kg diet	2867.48

[1] Vitamin and Mineral mixture (alfakema):- Each 1 kg contains:-Vit. A 580000 I.U, vit. D3 8600 I.U, vit. E. 720 mg, vit. K3 142 mg, vit C 0.1 mg, vit B1 58 mg, vit B2 34 mg, vit. B6 34 mg , vit.B12 58 mg , Folic acid 86 mg , Pantothenic acid 8 mg, Manganese sulfate 65 mg , Zinc methionine 3000 mg , Iron sulfate 2000 mg , Copper sulfate 3400 mg , Cobalt sulfate 572 mg , Sodium selenite 25 mg, Calcium iodide 25 mg, Calcium carbonate (Carrier substance) till one kg.

[2] According to **NRC (2011)**.

Fish rearing conditions and feeding regime

Healthy Nile tilapia fingerlings were obtained a live from Abbassa *fi*sh hatchery, Sharkia, Egypt. The fish did not have a history of disease outbreak and did not show any clinical abnormalities. Fish (27.56 ± 0.15 g) were randomly distributed into glass aquaria (10 fish/aquarium) in triplicates supplied with compressed air via air-stones for two weeks and fed on a basal diet (32.02% crude protein) before the beginning of the experiment. After the acclimation period, the fish were fed one of the tested diets two times per day at 9:00 and 2:00 h for 6 weeks until apparent satiation was reached.

The fish wastes were siphoned daily with half of the aquarium's water that is replaced by clean dechlorinated tap water. Water quality parameters were assessed and maintained within the allowed ranges of dissolved oxygen (6.08 ± 0.5 mg/L), pH (7.08

±0.13), unionized ammonia (0.31 ± 0.03 mg/L), and water temperature (29.55 ± 1.05 °C) with a controlled photoperiod (14 h light: 10 h darkness) in the laboratory. The protocol was approved by the Ethics of the Institutional Animal Care and Use Committee of Zagazig University, Egypt (ZU-IACUC–2-F–6–2020).

Growth performance and survival rate

The *fi*sh of each aquarium were weighed at the beginning (initial body weight) and the end of the trial (6 weeks) as final body weight. The growth parameters and survival rate (SR) were recorded according to **Jayant *et al*. (2018)** as follows;

Weight gain (WG, g) = Final body weight (g) – Initial body weight (g);
Daily WG = Total weight gain / Experimental days;
Specific growth rate (SGR, %/day) = 100 [(Ln Final weight – Ln Initial weight) / Number of days];
Feed conversion ratio (FCR) = Feed given (g) /Body WG (g);
Fish survival (SR; %) = 100 [Total number of fish harvested / Total number of fish stocked].

Sampling

At the end of the feeding period (6 weeks), blood samples from three fish from each replicate (9 fish/group) were collected using sterile syringes without anticoagulant and left at room temperature for 6 h to allow serum separation followed by centrifugation at 3000 rpm for 15 min. The serum samples were used to estimate immunological and biochemical parameters. Furthermore, samples of liver were taken and frozen at -20 ^{o} C for antioxidant enzymes analysis.

Serum immune assays

Lysozyme activity was detected using the lysoplate technique according to the previously described method **(Grinde, 1989)**. The level of serum nitric oxide (NO) was also assessed **(Moshage, 2009)**.

Serum biochemical assays

The serum total protein and albumin were determined spectrophotometrically **(Doumas *et al*., 1981; Doumas *et al*., ١٩٧١**, respectively**)**, while globulin was estimated by subtracting serum albumin from total protein **(Coles, 1986)**. Cortisol and glucose levels were evaluated by way of spectrophotometry, following the previous methods **(Burtis and Ashweed, 1999; Trinder, 1969,** respectively**)**. The liver (alanine (ALT) and aspartate (AST) aminotransferases) and kidney functions (creatinine and urea) using the Spinreact kits (Esteve De Bas, Girona, Spain) were measured according to **Burtis and Ashweed (1994), Fossati *et al*. (1983), and Kaplan (1984)**, respectively.

Hepatic antioxidant activity assays

The liver samples of three fish from each replicate (9 in total/group) were used to assess colorimetrically catalase (CAT) and superoxide dismutase (SOD) enzymes activities using commercial kits (Bio diagnostic Co., Cairo, Egypt) **(Aebi, 1984; McCord and Fridovich, 1969**, respectively**)**. Reduced glutathione (GSH) was also evaluated **(Beutler, 1963)**.

Bacterial challenge

A pathogenic *P. mirabilis* strain, previously isolated from diseased Nile tilapia at the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Egypt (unpublished data) was used in the challenge test. This isolate was identified by conventional biochemical tests, VITEK® 2 system (BioMérieux Inc., NC, USA) and 16S RNA (unpublished data). *P. mirabilis* was cultured onto tryptic soy agar (TSA) (Biolife, Milan, Italy) at 28 °C for 24 h. The lethal dose (LD50) of *P. mirabilis* was determined according to **Abdel-Razek *et al.* (2019)** and it was 10^7 CFU/mL and the sub lethal dose was used for the bacterial challenge.

At the end of the feeding trial (6 weeks), fish of each group were collected and randomly divided into two subgroups; each subgroup contained 10 fish in a 60-L aquarium. Fish of the first subgroup were intraperitoneally (IP) challenged with *P. mirabilis* using a sub lethal dose **(Schäperclaus *et al.*, 1992)** where a 0.1 mL of 24-h broth of *P. mirabilis* (5×10^6 CFU mL^{-1}). The second subgroup was IP injected with 0.1 mL of saline solution (control). Fasting fish was done 24 h before infection and the fish were resumed re-feeding on the corresponding diets 12h later. Fish were observed daily for 14 days to record mortalities, clinical signs, and postmortem findings. Re-isolation of *P. mirabilis* was done from moribund and freshly dead fish to confirm the specific mortality. At the end of the bacterial challenge (14 days), blood and liver samples were collected and all previous indices were measured. The relative percentage of survival (RPS) was also calculated according to the formula mentioned by **Amend (1981)**.

Statistical analysis

The normality of the obtained data was tested by using Kolmogorov–Smirnov's test and the homogeneity of the variance was tested by using Bartlett's test before statistical analysis. Then, the results were analyzed using one-way ANOVA to evaluate the effects of different feed supplements. Data of immune response, biochemical, and antioxidant parameters before and after the bacterial challenge were subjected to two-way ANOVA to explore the effects of feed supplements and bacterial infection as two factors. Duncan's multiple range tests were used to distinguish the differences between means at $P < 0.05$.

RESULTS

1. Growth performance and SR

The growth parameters of Nile tilapia fed on diets enriched with TP and/or CBP were presented in Table 2. Significant increases ($P < 0.05$) in the final body weight, feed intake, daily body WG, total body WG, and SGR and a significant decrease ($P < 0.05$) in the FCR value were observed in fish that received diets containing TP+CBP mixture followed by TP then CBP as compared with the control group. The SR did not significantly alter by TP and/or CBP dietary enrichment.

Table 2. The growth performance and survival rate of Nile tilapia fed on turmeric powder (TP) and/or clove bud powder (CBP) enriched diets for 6 weeks.

Parameters	Experimental groups			
	Control	**TP**	**CBP**	**TP + CBP**
IBW/fish (g)	27.73 ±0.23	27.43 ±0.31	27.60 ±0.36	27.50 ±0.43
FBW/fish (g)	42.93 ±0.94 ^{d}	51.33 ±0.53 ^{b}	47.53 ±0.26 ^{c}	55.96 ±0.31^{a}
Daily BWG/ fish(g)	0.33 ±0.02 ^{d}	0.53 ±0.01 ^{b}	0.44 ±0.003 ^{c}	0.63 ±0.006 ^{a}
Total BWG/ fish(g)	15.20 ±1.15 ^{d}	23.90 ±0.80 ^{b}	19.93 ±0.14 ^{c}	28.46 ±0.34 ^{a}
Total FI/fish (g)	50.43 ±1.10 ^{d}	60.30 ±0.63 ^{b}	55.84 ±0.30 ^{c}	65.74 ±0.37 ^{a}
SGR (%/day)	0.97 ±0.06 ^{d}	1.39 ±0.04 ^{b}	1.20 ±0.01^{c}	1.57 ±0.02 ^{a}
FCR	3.34 ±0.16 ^{a}	2.52 ±0.06 ^{bc}	2.80 ±0.03 ^{b}	2.31 ±0.02 ^{c}
SR	100 ±0.0	100 ±0.0	100 ±0.0	100 ±0.0

Control group = Fish fed basal diet without any feed addition; TP group = Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture of 0.5% TP and 3% CBP. IBW, Initial body weight; FBW, Final body weight; BWG, Bodyweight gain; FI, feed intake; SGR, Specific growth rate; FCR, Feed conversion ratio; SR, survival rate. Mean values in the same row with different superscript differ significantly ($P < 0.05$).

2. Serum immune indices

The two-way ANOVA revealed that levels of serum lysozyme and NO were significantly affected ($P < 0.05$) by dietary TP and CBP enrichment, bacterial infection, and their interaction (Table 3). These immune parameters were markedly elevated by the dietary addition of TP and/or CBP, where their highest levels were observed at fish fed TP+CBP mixture. After the bacterial infection, these immune parameters were significantly augmented ($P < 0.05$) in fish fed a diet enriched with TP+CBP mixture followed by TP then CBP as compared with the control group, however, their levels were lower than before bacterial infection.

3.Serum protein profile, cortisol, and glucose levels

Table 3. Serum immune parameters of Nile tilapia fed on turmeric powder (TP) and/or clov bud powder (CBP) enriched diets for 6 weeks and after bacterial challenge for 14 days.

Groups	Lysozyme (µg mL^{-1})	Nitric oxide (µmol L^{-1})
Before infection		
Control	17.00 ±0.57 ^{g}	36.80 ±0.05 ^{f}
TP	35.66 ±0.88 ^{b}	87.70 ±0.05 ^{b}
CBP	31.77 ±0.28 ^{c}	77.23 ±0.06 ^{c}
TP+ CBP	42.00 ±0.57 ^{a}	91.30 ±0.05 ^{a}
After infection		
Control	9.66 ±0.33 ^{h}	20.40 ±0.05 ^{g}
TP	21.51 ±0.28 ^{e}	37.10 ±0.05 ^{e}
CBP	19.36 ±0.09 ^{f}	36.63 ±0.08 ^{f}
TP+ CBP	28.83 ±0.28 ^{d}	38.14 ±0.09 ^{d}
Two-way ANOVA	***P* value**	
Feed supplements	0.0001	0.0001
Bacterial infection	0.0001	0.0001
Feed supplements x bacterial infection	0.0001	0.0001

Control group= Fish fed basal diet without any feed addition; TP group= Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture 0.5% TP and 3% CBP. Mean values in the same row with different superscript differ significantly ($P < 0.05$).

As cleared in Table 4, it was noticed that total protein, albumin, globulin, cortisol, and glucose levels were significantly affected ($P < 0.05$) by dietary TP and/or CBP and bacterial infection except for the serum albumin that was not affected by dietary TP and/or CBP. There were significant interaction effects of dietary TP and/or CBP and bacterial infection in the above-mentioned parameters except for globulin and cortisol levels.

Dietary TP and/or CBP significantly augmented ($P < 0.05$) total protein and globulin, while albumin was reduced except for CBP fed group as compared with the control fish (Table 4). After bacterial infection, these parameters were significantly elevated ($P < 0.05$) in fish fed TP+CBP mixture followed by TP then CBP as compared with the control group; however, their levels were lower than before bacterial infection. Serum cortisol was not significantly altered by dietary inclusion of TP and/or CBP; however glucose level was significantly reduced (Table 4). After bacterial challenge, dietary TP+CBP mixture followed by TP then CBP significantly decreased ($P < 0.05$) cortisol and glucose levels as compared to the control fish, however, their levels were higher than before bacterial infection.

Table 4. Serum protein profile and stress indicators of Nile tilapia fed on turmeric powder (TP) and/or clove bud powder (CBP) enriched diets for 6 weeks and after bacterial challenge for 14 days.

Groups	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Total globulin (g dL^{-1})	Cortisol (mg dL^{-1})	Glucose (mg dL^{-1})
Before infection					
Control	6.09 ±0.06 ^{b}	2.87 ±0.36 ^{ab}	3.22 ±0.33 ^{de}	0.96 ±0.29 ^{bc}	71.13 ±0.19 ^{d}
TP	7.56 ±0.005 ^{a}	2.64 ±0.34 ^{bc}	4.91 ±0.33 ^{ab}	0.71 ±0.003 ^{bc}	56.33 ±0.33 ^{f}
CBP	7.42 ±0.01 ^{a}	3.47 ±0.22 ^{a}	3.94 ±0.22 ^{cd}	0.77 ±0.006 ^{bc}	60.66 ±0.33 ^{e}
TP+ CBP	7.54 ±0.006 ^{a}	2.55 ±0.43 ^{bcd}	4.98 ±0.43 ^{a}	0.69 ±0.006 ^{c}	55.33 ±0.33 ^{f}
After infection					
Control	2.41 ±0.14 ^{e}	1.28 ±0.18 ^{e}	1.13 ±0.32 ^{f}	1.98 ±0.005 ^{a}	105 ±0.11 ^{a}
TP	5.54 ±0.006 ^{c}	2.17 ±0.10 ^{bcd}	3.37 ±0.005 ^{de}	1.13 ±0.01 ^{bc}	74.66 ±0.33 ^{c}
CBP	4.38 ±0.006 ^{d}	1.76 ±0.01 ^{de}	2.62 ±0.008 ^{e}	1.18 ±0.006 ^{b}	77.00 ±0.57 ^{b}
TP+ CBP	6.14 ±0.03 ^{b}	2.02 ±0.03 ^{cd}	4.12 ±0.005 ^{bc}	1.16 ±0.46 ^{b}	70.33 ±0.33 ^{d}
Two-way ANOVA			***P* value**		
Feed supplements	0.0001	0.230	0.0001	0.003	0.0001
Bacterial infection	0.0001	0.0001	0.0001	0.0001	0.0001
Feed supplements x bacterial infection	0.0001	0.041	0.182	0.131	0.0001

Control group= Fish fed basal diet without any feed addition; TP group= Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture of 0.5% TP and 3% CBP. Mean values in the same row with different superscript differ significantly ($P < 0.05$).

4. Serum hepato-renal indicators

The levels of serum ALT, AST, creatinine, and urea were significantly affected ($P < 0.05$) by dietary TP and CBP enrichment, bacterial infection and their interaction except for urea level that was not affected by interaction (Table 5). Dietary TP and/or CBP inclusion did not affect the levels of these parameters. After the bacterial challenge, these parameters were significantly declined ($P < 0.05$) in groups fed on TP+CBP

mixture followed by TP then CBP, however, their levels were higher than before bacterial infection.

Table 5. Hepato-renal function indicators of Nile tilapia fed on turmeric powder (TP) and/or clove bud powder (CBP) enriched diets for 6 weeks and after bacterial challenge for 14 days.

Groups	ALT (U L^{-1})	AST (U L^{-1})	Creatinine (mg d L^{-1})	Urea (mg d L^{-1})
Before infection				
Control	13.16 ±1.04 ^{d}	17.13 ±1.34 ^{d}	0.66 ±0.16 ^{c}	15.07 ±3.32 ^{b}
TP	12.16 ±1.18 ^{d}	16.96 ±1.57 ^{d}	0.63 ±0.17 ^{c}	14.17 ±3.07 ^{b}
CBP	12.83 ±0.72 ^{d}	16.47 ±1.65 ^{d}	0.63 ±0.16 ^{c}	14.94 ±3.29 ^{b}
TP+ CBP	12.76 ±1.29 ^{d}	15.20 ±0.15 ^{d}	0.62 ±0.15 ^{c}	14.96 ±3.32 ^{b}
After infection				
Control	38.51 ±0.15 ^{a}	35.83 ±0.03 ^{a}	1.99 ±0.005 ^{a}	28.60 ±0.06 ^{a}
TP	20.23 ±0.15 ^{b}	29.16 ±0.12 ^{b}	1.12 ±0.01 ^{b}	17.13 ±0.09 ^{b}
CBP	22.30 ±0.16 ^{b}	33.16 ±0.08 ^{a}	1.20 ±0.003 ^{b}	18.03 ±0.03 ^{b}
TP+ CBP	18.91 ±0.04 ^{c}	25.50 ±0.02 ^{c}	0.98 ±0.005 ^{bc}	16.40 ±0.05 ^{b}
Two-way ANOVA		***P* value**		
Feed supplements	0.0001	0.0001	0.002	0.046
Bacterial infection	0.0001	0.0001	0.0001	0.005
Feed supplements x bacterial infection	0.0001	0.002	0.002	0.066

Control group= Fish fed basal diet without any feed addition; TP group= Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture of 0.5% TP and 3% CBP. ALT, Alanine aminotransferase; AST, Aspartate aminotransferase. Mean values in the same row with different superscript differ significantly ($P < 0.05$).

5. Hepatic antioxidant indices

It was noticed that CAT, SOD, and GSH levels were significantly affected ($P < 0.05$) by dietary TP and/or CBP and bacterial infection and their interaction as presented in Table 6. The highest antioxidant levels of these parameters were recorded in fish fed dietary TP+CBP mixture followed by TP then CBP as compared with the control group, however, no significant difference was recorded in the GSH level between fish that received TP+CBP mixture and TP. After bacterial infection, these parameters were significantly augmented ($P < 0.05$) in all TP and/or CBP; however, their levels were lower than before bacterial infection.

Table 6. Hepatic antioxidant parameters of Nile tilapia fed on turmeric powder (TP) and/or clove bud powder (CBP) enriched diets for 6 weeks and after bacterial challenge for 14 days.

Groups	CAT (U g^{-1} tissue)	SOD (U g^{-1} tissue)	GSH (mmol g^{-1} tissue)
Before infection			
Control	15.63 ±0.05 ^{d}	5.60 ±0.05 ^{d}	1.30 ±015 ^{c}
TP	22.00 ±0.57 ^{b}	9.06 ±0.03 ^{b}	2.76 ±0.003 ^{a}
CBP	20.33 ±0.33 ^{c}	8.11 ±0.003 ^{c}	2.45 ±0.005 ^{b}
TP+ CBP	25.00 ±0.57 ^{a}	9.24 ±0.03 ^{a}	2.87 ±0.03 ^{a}
After infection			
Control	7.23 ±0.05 ^{g}	1.17 ±0.07 ^{h}	0.57 ±0.005 ^{e}
TP	12.44 ±0.21 ^{f}	4.36 ±0.005 ^{f}	0.94 ±0.005 ^{d}
CBP	11.66 ±0.06 ^{f}	3.44 ±0.06 ^{g}	0.91 ±0.003 ^{d}
TP+ CBP	14.10 ±0.05 ^{e}	5.43 ±0.005 ^{e}	0.98 ±0.003 ^{d}
Two-way ANOVA		***P* value**	
Feed supplements	0.0001	0.0001	0.0001
Bacterial infection	0.0001	0.0001	0.0001
Feed supplements x bacterial infection	0.006	0.001	0.0001

Control group= Fish fed basal diet without any feed addition; TP group= Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture of 0.5% TP and 3% CBP. CAT, catalase; SOD, superoxide dismutase; GSH, reduced glutathione. Mean values in the same row with different superscript differ significantly ($P < 0.05$).

6. P. mirabilis challenge

The fish in all groups showed normal signs and behavior during the feeding trial. After challenge with *P. mirabilis*, the control fish that did not receive TP and/or CBP in diets exhibited off food, sluggish movement, and loss of scales; sever fin rot and hemorrhages on body surfaces, and skin ulcerations (Figure 1 A and B) with congested internal organs including gills and enlarged liver (Figure 1 C). These signs and lesions were highly improved in fish that received TP and /or CBP in diets.

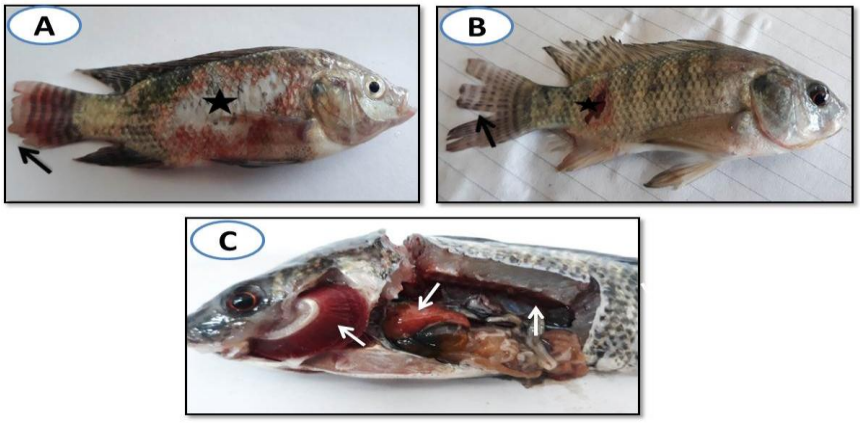

Figure 1. Clinical signs and post-mortem findings of *Proteus mirabilis* infection in the Nile tilapia that fed basal diet without any feed addition. A and B: Fish showed skin darkness, severe body hemorrhages (A), fin rot (black arrow), and skin ulceration (star). C: Fish showed congested internal organs including gills and enlarged liver (white arrows).

As cleared in Table 7, the highest mortality rate after the bacterial infection was found in the control group (40%) and this rate was declined gradually to be 30, 10, and 10% in fish fed CBP, TP, and TP+CBP mixture enriched diets, respectively. Dietary TP and/or CBP improved the fish resistance to *P. mirabilis*, where the highest RPS was at the fish fed TP+CBP mixture and TP (75%).

Table 7. The mortality rate % and relative percentage survival (RPS) of Nile tilapia fed on turmeric powder (TP) and/or clove bud powder (CBP) enriched diets after challenged with *Proteus mirabilis* for 14 days.

Groups	Mortality (%)	Survival (%)	Relative percentage of survival (%)
Control	40	60	-
TP	10	90	75
CBP	30	70	25
TP+ CBP	10	90	75

Control group= Fish fed basal diet without any feed addition; TP group= Fish fed basal diet enriched with 0.5% TP; CBP group= Fish fed basal diet enriched with 3% CBP; TP + CBP group= Fish fed basal diet enriched with a mixture of 0.5% TP and 3% CBP.

DISCUSSION

Phytobiotics can serve as a natural alternative to antibiotics and also can augment fish immune response in facing emerging diseases. The growth performance is a good health indicator and it can be estimated as the change in fish weight, length, and size. In the present study, the growth performance and feed intake of Nile tilapia that fed on diets enriched with TP and/or CBP for 6 weeks were enhanced and the highest growth rate was recorded in the combination group. The improved growth with dietary TP could be returned to the enhanced feed consumption and utilization through the promotion of digestive enzymes activity **(Prasad and Aggarwal, 2011; Yitbarek, 2015)**. Also, TP increased intestinal villous heights and goblet cell numbers **(Kaur *et al*., 2020)**, indicating the increased surface area for the absorption of nutrients **(Kurhekar, 2013)**. Moreover, the clove is a rich source for antioxidants that can prevent lipid peroxidation and oxidative rancidity, which declines the nutritional value of fat and other feed components **(Gaber, 2000)**. All these beneficial effects could increase the growth and improve the fish general health **(Chakraborty *et al*., 2014)**. The improved growth of Nile tilapia fed dietary TP at a dose of 0.5 or 4% **(Mahmoud *et al*., 2014; Sanchez *et al*., 2019)**, respectively, or fed dietary clove oil at a dose of 0.08% **(Gaber, 2000)** was reported.

In the present study, *P. mirabilis* infection in Nile tilapia badly affected the different measured parameters. Although, there were no available literature about the effect of *P. mirabilis* on fish, this could be attributed to that *Proteus mirabilis* possess the virulence factors of the pathogenic enteric bacteria like somatic antigens, extra-cellular protein, adhesins, colicins, and lipopolysaccharides that can pierce the intestinal mucous cells and facilitate pathogenicity and disease process **(Stańkowska *et al*., 2008; Różalski, 2012)**. Also, **Belas *et al*. (2004)** reported that proteases of *P. mirabilis* can degrade other types of biologically important proteins resulting in immunosuppression

The immunomodulation is a vital management tool in aquaculture for disease protection **(Abdel Rahman *et al*., 2018b and 2019)**. The bacterial challenge test is used to confirm the immunomodulatory effect of TP and CBP either alone or in combination.

Lysozyme is an antimicrobial enzyme produced by leucocytes that lysis the cell wall of bacteria and initiates phagocytosis **(Saurabh and Sahoo, 2008)**. Strongly bactericidal reactive oxygen named NO is released from the macrophages to enhance its ability in killing pathogens **(Neumann *et al*., 2001)**. Serum proteins are considered a prime indicator of fish health and their high values especially globulin associated with potent fish immune response **(Asadi *et al*., 2012)**.

In the present study, lysozyme, NO, and total protein especially globulin levels were markedly elevated by the dietary addition of TP and/or CBP, where their highest levels were observed at combination group before and after the bacterial infection. These positive effects could reduce fish susceptibility to *P. mirabilis* infection and this was confirmed by the elevation of RPS after bacterial challenge. Such a modulation that was resulted in the present study, could be due to the active constituents present especially curcumin in TP that has a powerful immunomodulatory action through its effect on antigen presentation, lymphoid cell populations, cytokine production **(Varalakshmi *et al*., 2008; Sankar *et al*., 2010)**. Also, the eugenol in CBP can act as an enhancer to lymphocyte proliferation and macrophage production **(Halder *et al*., 2011; Wael *et al*., 2018)**. The synergistic effect of both TP and CBP was more efficient in stimulating the fish immune response. The immunostimulatory and disease protection effect of TP demonstrated herein are agreed with previous reports in Nile tilapia **(Abdelrazek *et al*., 2017; Ayoub *et al*., 2019)**, common carp **(Abdel-Tawwab and Abbass, 2017)**, African catfish, *Clarias gariepinus* **(Adeshina *et al*., 2017)**, and rohu, *Labeo rohita* **(Kaur *et al*., 2020)**. Similar improvements in mortality rate by clove oil and extract were reported in Nile tilapia challenged with *Lactococcus garvieae* **(Rattanachaikunsopon and Phumkhachorn, 2009)** and African catfish challenged with *Aeromonas sobria* **(Ghaly *et al*., 2015)**.

In fish, blood indices can act as an important indicator of health and stressful conditions **(Wagner and Congleton, 2004)**. During stress, the elevated level of cortisol results in an elevation in glucose levels to provide the energy to withstand unfavorable conditions **(Olusola and Nwokike, 2018)**. In the present study, dietary TP and/or CBP did not affect serum cortisol but lowered blood glucose levels. These two parameters were decreased after bacterial challenge indicating that TP and CBP could mitigate the infection-induced stress. These effects might be attributed to the suppression of serotonin production by TP that leads to a decrease in cortisol release **(Xia *et al*., 2007)**. A previous decline in cortisol and glucose of common carp fed on 1,8-cineol (an active compound of turmeric) during water ammonia exposure **(Mirghaed *et al*., 2019)** or TP during copper exposure **(Rajabiesterabadi *et al*., 2020)** and of rainbow trout, *Oncorhynchus mykiss* fed on 1,8-cineol during crowding stress **(Mirghaed *et al*., 2018)** was reported. Also, these parameters were declined in common carp exposed to CBP as an anesthetic agent at low water temperature **(Abdulrahman *et al*., 2018)** and in rohu exposed to clove oil **(Devi and Kamilya, 2019)**.

ALT and AST are the most important index of liver diseases and the hepatic damage results in their elevations in fish **(Javed *et al*., 2016)**. An elevation in the serum creatinine and urea is also an indicator of nephrotoxicity **(Cengiz, 2006)**. The present study showed dietary TP and/or CBP inclusion did not affect ALT, AST, creatinine, and urea levels; however, these parameters were declined after bacterial infection. Such decline might be due to the hepato and nephro-protective effects of TP and CBP

(Alambra *et al*., 2012; Kaur *et al.*, 2020) owing to their potent antioxidant properties **(Hussein *et al*., 2014)**. A reduction in these parameters was previously recorded in common carp **(Hoseini *et al*., 2018; Rajabiesterabadi *et al*., 2020)** and rohu **(Kaur *et al*., 2020)** that fed on TP supplemented diets. Dietary TP could restore these parameters within the normal values as the control fish in African catfish exposed to cadmium toxicity **(El-Houseiny *et al*., 2019)**.

The antioxidant system of the fish is a type of defense mechanism that protects the body tissues from oxidative stress **(Saglam *et al*., 2014)**. In the present study, notably, the dietary TP+CBP mixture incorporation followed by TP and CBP were seen to markedly increase the hepatic levels of CAT, SOD, and GSH before and after bacterial infection. The antioxidant capability of TP may be returned to its potent content of curcumin, ascorbic acid, and 1,8-cineole that can exhibit a strong free radicals action **(Singh *et al*., 2010; Dosoky *et al*., 2019)**. Moreover, the powerful antioxidant activity of clove could be attributed to its high content of eugenol, eugenol acetate, and thymol **(Gülçin *et al*., 2012; Nam and Kim, 2013)**. Previous reports confirmed the antioxidant properties of TP in Nile tilapia exposed to aflatoxin **(Mahfouz, 2015)** and rainbow trout challenged with *A. salmonicida* **(Yonar *et al*., 2019)**. Also, the SOD of rohu fed on dietary TP and challenged with *A. veronii* was markedly elevated **(Kaur *et al*., 2020)**. In rats, the phenolic compounds of clove oil exerted an antioxidant effect that could protect the liver from aflatoxin **(Abdel-Wahhab and Aly, 2005)**.

CONCLUSION

The present study evoked that the dietary TP and/or CBP could improve growth, immune response, antioxidant status of Nile tilapia. It could also protect fish from pathological alterations resulted from *P. mirabilis* infection. Therefore, both supplements might be used as immune-stimulants and antimicrobial agents without any adverse effects on fish, and their combination is more beneficial than each supplement alone.

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