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Effect of Turmeric (Curcuma longa) Black Pepper (Piper nigrum) and Azolla Pinnata on Waste Water Nile tilapia Fish

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Abstract

The objective of this study was to investigate the effect of Turmeric (Curcuma longa) with Black Pepper (Piper nigrum) and Azolla Pinnata on Nile tilapia fish weight and the gene expression of growth hormone receptor (GHR) and insulin as growth factor (IGF) of Nile tilapia collected from waste water. A total of 250 Nile tilapia fish divided into 5 groups each of 50. The first group of apparently healthy fish collected from Central Laboratory for Aquaculture Research El-Abbassa (clean water). The second group collected from waste water of Bahr El-Baqar zone. The third group collected from waste water of Bahr El-Baqar zone and fed on 0.5% (5g/kg diet) of each Turmeric (Curcuma longa) and Piper nigrum for 45 days. The fourth group collected from waste water of Bahr El-Baqar zone in the presence of small amount of Azolla Pinnata (Aquatic fern) for 45 days (Azolla grows quickly without feeding on Turmeric and Piper nigrum), while the fifth group collected from waste water of Bahr El-Baqar zone and fed on Turmeric (Curcuma longa) and Piper nigrum for 45 days in presence of Azolla Pinnata. The results cleared that the weight of fish in the first group showed an increase after 45 days but a slight decrease in weight were noticed in the second group in the first 15 days. There was an increase in fish weights in third and fourth groups and a marked increase in the fifth group after 45 days. Moreover, an increase in both GHR and IGF in groups fed on Turmeric and Piper nigrum for 45 days as well as in the presence of Azolla Pinnata.

Keywords: Turmeric, Curcuma longa, Piper nigrum, Azolla, Waste Water, Nile tilapia.

Introduction

Heavy metals in waste water have an effect on fish health by accumulation in their organs especially gills and liver by a quantity more than muscles [1]. Bioaccumulation of heavy metals in fish may critically affects physiological and biochemical status and the growth rate of fish [1]. The aquatic environment and water quality are the main factors that control the state of health and disease in both cultured and wild fish [2]. Bahr El-Baqer is considered as one of polluted regions because its drains contain both industrial and domestic waste water that have an effect on Lake Manzala [3]. It was important to use plants in therapy "back to nature" to avoid using of synthetic drugs that have dangerous side effects [4]. Curcumin is considered as the active ingredient from the spice turmeric that has a potent antioxidant effect [5]. It has hepato protective properties and also it is strongly alleged that turmeric has ability to improve nutrient and digestion metabolism [6].

Black pepper has immunomodulatory roles and they can be used as natural agents that can promote the maintenance of a healthy immune system as it plays a role in growth enhancement [7]. Moreover, Curcumin's absorption was enhanced when black pepper is combined with it, whereas black pepper can enhance the blood concentration of fish that extended the absorption and bioavailability of curcumin so it is recommended that adding black pepper to curcumin [8]. Azolla can grow quickly and they can act as symbiont due to presence of Anbaena group [9]. It also acts as a biofilter to purify water and can remove phosphorus and nitrogen. In addition, it can remove sulfa drugs [10] as well as it removes metals such as Cd, Cr, Pb, Au, Sr, Cu and Zn [11]. This study aimed to investigate the effect of both Turmeric (Curcuma longa) with Black Pepper (Piper nigrum) and Azolla Pinnata on Nile tilapia that was treated in waste water to avid the dangerous resulted from waste water.

Materials and Methods

Fish

Fifty Nile tilapia were obtained from the Central Laboratory for Aquaculture Research El-Abbassa, Abo Hammad, Sharkia. These fish were analyzed and had permissible limits of copper and zinc in muscles (6.48±0.03 and

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44.12±0.05 mg/g, respectively) and gills (9.41±0.098 and 81.64±0.056 mg/g, respectively). There wasn't cadmium in muscles but it was in gills (9.41±0.098 mg/g), while iron in muscles and gills were 82.61±0.029 and 214.74±0.042 mg/g), so they were used in the current study as a negative control (Group 1). Two hundred Nile Tilapia were obtained from Bahr El-baqar zone, Alhusiniuh, Sharkia. These fishes were analyzed and had limits of copper in muscles and gills (7.46±0.23 and 11.32±0.03 mg/g, respectively), while, zinc and cadmium in muscle was 66.72±0.08 and 0.02±0.003 mg/g, respectively) and in gills was 90.17±0.05 and 11.32±0.03 mg/g, respectively). Iron in muscles and gills was 145.45±0.007 and 310.46±0.05 mg/g, respectively and they were used in current study as positive control (Group 2). All fish were maintained in concrete tanks and their average body weight was 70±10 g.

Experimental design

Nile tilapia were collected from Central Laboratory for Aquaculture Research El-Abbassa (fresh water with salinity less than 0.05%, dissolved oxygen 5.1±0.6 mg|L, PH 7.5, total alkalinity 442.99±11.9, total ammonia 0.55 ± 0.07 mg|L, unionized ammonia 0.07 ± 0.001 mg|L, Nitrite 0.09 ± 0.0002mg|L, nitrate 0.35±0.005 mg|L and 26ºC) they were fed on ingredients of feeding diet without additives. These fishes were used as a negative control group (Group 1).

The 200 Nile tilapies from Baher El Baker (with salinity more than 0.05%, dissolved oxygen 2.12±0.5 mg|L, PH 7.3, total alkalinity 453.35 -8.7, Total ammonia 39.40 ± 1.9 mg|L , Unionized ammonia 7.02 ± 1.2 mg|L, Nitrite 0.04±0.08 mg|L, nitrate 0.25±0.55 mg|L and 26ºC) were divided into four groups. The first was kept as the same as positive control (Group 2). Group 3: Nile tilapia from Baher El Baker that were fed on ingredients of feeding diet with addition of Turmeric powder 0.5% (5g/kg diet) [12-14] and 0.5% Piper nigrum [15] (Table1). Group 4: Nile tilapia from Baher El Baker were exposed to Azolla Pinnata (Aquatic fern) with small amount as Azolla grows quickly [7], and were fed on ingredients of feeding diet without additives (Table 1). Group 5: Nile tilapia from Baher El Baker were exposed to Azolla Pinnata (Aquatic fern) and fed on ingredients of feeding diet with addition of turmeric powder and piper nigrum (Tables 1,2).

All groups were observed along 45 days and were maintained in concrete aquariums with a capacity of 10597.5 Liters, but fish were cultivated in 8831.25 Liters. Nile tilapias were fed twice daily with 5% from total weight of fish for every aquarium. Fish were individually weighed to the nearest 70±10 gram at the beginning of the experiment using a digital balance (Model Ainsworth A-600) and also every 15 days throughout the experimental period [16]. Liver samples were collected from Nile tilapia fish and used immediately for determination of GHR and IGF gene expression using RNeasy Mini Kit (QIAGEN, USA).

Table (1): Ingredients of feeding diet for Nile Tilapia [15,34].

Ingredients	Level of ingredients	
	Traditional diet	Additional diet
Fish meal	10.4%	10.4%
Soybean meal (SBM)	42.98%	42.98%
Ground corn (CNM)	20.32%	20.32%
Wheat bran (WB)	15.49%	15.49%
Cod fish oil	2.31%	2.31%
Corn oil	1.50%	1.50%
Vitamins Premix	1.0%	1.0%
Minerals Premix	2.0%	2.0%
Starch	4.0%	4.0%
Turmeric powder	———	0.5%
Piper nigrum	———	0.5%

Table 2: Experimental design of different treatments of waste water fish.

Groups	Water	Treatments	
		Diet treatment	Water treatment
G_1	Fresh	Traditional diet	_____
G_2	Waste	Traditional diet	_____
G_3	Waste	Additional diet	_____
G_4	Waste	Traditional diet	Exposure to azolla
G_5	Waste	Additional diet	Exposure to azolla

G_1: (negative control group): fish from Central Laboratory for Aquaculture Research EL-Abbassa (clean water); G_2: (positive control group) fish that were cultivated in waste water; G_3: fish from waste water that were treated with Turmeric (Curcuma longa) with black pepper (Piper nigrum); G_4: fish from waste water that were exposure to Azolla and G_5: fish from waste water that were treated with both Turmeric (Curcuma longa) with black pepper (Piper nigrum) and Azolla.

Gene expression analysis using Quantitative Real Time PCR

Growth Hormone Receptor (GHR): (5′-CAG ACT TCT ACG CTC AGG TC-3′), (5′-CTG GAT TCT GAG TTG CTG TC-3′). Amplicon (80 bp): Gene Bank (ID): AY973232.1 and Insulin as growth factor (IGF): (5′-GTTTGTCTGTGGAGAGCGAGG-3′), (5′-GAAGCAGCACTCGTCCACG -3′). Amplicon (bp): 97 and Gene Bank (ID): Y10830.1

Quantitative Real Time PCR (qRT-PCR)

Triplicate PCR reactions were done for each analyzed sample. Each PCR reaction consisted of, 2.5 µL of 1µg/µL cDNA, 12.5 µL SYBR Green PCR Master Mix, 0.3 M of both forward and reverse primer and double distilled water to a final volume of 25 µL. Reactions were analyzed on an Applied Biosystem 7500 Real time PCR detection system under the following conditions: 95°C for 10 min and 45 cycles of 95°C for 20s then 60°C for 20s and 72°C for 20s. The fluorescence monitoring takes place at the end of each cycle. 18s rRNA gene can used as reference gene for qPCR data normalization [17].

Statistical analysis

All experimentally induced changes in IGF and GHR expression were presented as n-fold changes relative compared to controls set as 1 (100%). The comparative threshold cycle (ΔΔCT) method [18] was used to calculate relative gene expression ratios [19]. Prior to analysis, qPCR assays were validated by plotting CT values against the logarithms of the dilution factors. Relative gene expression ratios (R) between treated and control groups were calculated using the formula: $R=2^{-\Delta\Delta CT}$ with ΔCT = CT (target gene) -CT (reference gene), with $\Delta\Delta CT = \Delta CT$ (treated group) - ΔCT (untreated control). All data are presented as means ± standard error (SE) and were analyzed using one way ANOVA, followed by multiple range tests was used to compare differences among individual means [20], with statistical software SAS ANOVA procedure (Statistical Analysis System, 1993). A probability of 0.05 was utilized to account for the statistical difference among means.

Results

The trials of water and diet treatments of fish to avoid the bad impact of cultivation of fish in waste water revealed the following results. The initial weight was the same for all five groups (73.37±0.87). The follow up fish weight changes in five groups showed that fish weight after 15 days from the experiment was 88.73±1.2, 72.04±0.86, 72.94±0.86, 72.84±0.87 and 73.00±0.88 for the five groups respectively. Nile tilapia fish weight at the end of the experiment was 126.13±1.44, 79.07±0.90, 109.64±0.51, 102.0±0.89 and 116.84±0.37 (Table 3). It was noticed an improvement in groups that were treated with turmeric, black pepper and azolla as compared with the group 2.

Table 3: Fish weight in different groups at different periods.

Treatment	Initial weight of fish	Weight of fish after 15 days	Weight of fish after 45 days
G_1 (g)	73.096±1.23	88.728±1.20[a]	126.13±1.44[a]
G_2 (g)	73.374±0.87	72.042±0.856[b]	79.07±0.90[e]
G_3 (g)	73.374±0.87	72.941±0.855[b]	109.64±0.51[c]
G_4 (g)	73.374±0.87	72.844±0.867[b]	102.0±0.89[d]
G_5 (g)	73.374±0.87	73.002±0.877[b]	116.48±0.37[b]

G_1: (negative control group): fish from Central Laboratory for Aquaculture Research EL-Abbassa (clean water); G_2: (positive control group) fish that were cultivated in waste water; G_3: fish from waste water that were treated with Turmeric (Curcuma longa) with black pepper (Piper nigrum); G_4: fish from waste water that were exposure to Azolla and G_5: fish from waste water that were treated with both Turmeric (Curcuma longa) with black pepper (Piper nigrum) and Azolla. Means carrying different superscripts (letters) within the same column were significantly differences at ($P<0.05$).

The liver tissue showed a highly significant increase in the level of gene expression activity of growth hormone receptor gene of group 3 that was treated with turmeric and black pepper also group 4 that was treated with Azolla and group 5 that was treated with both turmeric, black pepper and Azolla as compared with both positive and negative control groups (Table 4). The detection of level of gene expression activity of insulin as growth factor in liver tissue showed significant increase in group 3 that was treated with turmeric and black pepper also in group 4 that was treated with Azolla as well as in group 5 that was treated with both turmeric, black pepper and Azolla as compared with both positive and negative control groups (Table 5).

Table 4: Analysis of PCR product of Growth hormone receptor gene in liver tissue of Nile tilapia (*Oreochromis niloticus*)

Group	Fold Change Mean±Standard Error)
G1	1.06606±0.149186
G2	0.547623±0.19069
G3	2.45439±0.237686
G4	0.777176±0.498806
G5	3.696522±0.437488

G_1: (negative control group): fish from Central Laboratory for Aquaculture Research EL-Abbassa (clean water); G_2: (positive control group) fish that were cultivated in waste water; G_3: fish from waste water that were treated with Turmeric (Curcuma longa) with black pepper (Piper nigrum); G_4: fish from waste water that were exposure to Azolla and G_5: fish from waste water that were treated with both Turmeric (Curcuma longa) with black pepper (Piper nigrum) and Azolla.

Discussion

Turmeric supplementation has the ability to increase the growth rate due to improve feed utilization and feed consumption, which are considered as an indicator of increasing nutrient digestibility of turmeric [21]. Moreover, Turmeric can stimulate protein synthesis through enzymatic system [22]. As well as piper nigrum has an ability to control the resistant bacteria which are becoming a threat to human health [23] and can be used as natural agents that promote the maintenance of a healthy immune system and therefore, improve fish weight [7]. Azolla is rich in protein (25-30%) [24] and has the ability to improve fish weight. There was significant increase in weight of fish that treated with Curcuma longa and Piper nigrum or azolla and that were treated by turmeric powder, black

pepper and azolla (Group 5). While, a decrease in weight of fish collected from waste water due to the impact of heavy metals on fish weight.

Table 5: Analysis of PCR product of Insulin gene in liver tissue of Nile tilapia (Oreochromis niloticus)

Group	Fold Change Mean±Standard Error)
G1	1.1404±0.270337
G2	0.527377±0.324043
G3	0.67543±0.0903657
G4	0.571434±0.5594856
G5	0.687961±0.04285

G_1: (negative control group): fish from Central Laboratory for Aquaculture Research EL-Abbassa (clean water); G_2: (positive control group) fish that were cultivated in waste water; G_3: fish from waste water that were treated with Turmeric (Curcuma longa) with black pepper (Piper nigrum); G_4: fish from waste water that were exposure to Azolla and G_5: fish from waste water that were treated with both Turmeric (Curcuma longa) with black pepper (Piper nigrum) and Azolla.

The growth in fish is depended on growth hormone that was secreted from the pituitary gland and regulates the somatic growth in addition to organ and tissue growth [25]. These biological functions are depended on insulin like growth factor that released from the liver in response to circulating growth hormone [26]. Analysis of PCR in liver tissues of Nile tilapia showed an increase in the activity of gene expression of both growth hormone receptor (GHR) gene and insulin like growth factor gene (IGF) in Nile tilapia *(Oreochromis niloticus)* fish that were treated by turmeric powder, black pepper or azolla and that treated with both turmeric powder and black pepper and azolla. Curcumin is considered as a prebiotic that can improve the balance of positive and negative intestinal flora [27] and it can increase the intestinal digestion and absorption that leads to stimulate the growth and improve the general health of fish [25]. The current results are in agreement with Midhun *et al*. [28] who reported an increase in GH, GHR and IGF when curcumin has been used as feed additive to *tilapia* for 45 days. Rojtinnakorn *et al*. [29] showed that all turmeric extract fed fish had significant higher activities of digestive enzymes because it considered as an indicator on improvement of growth rate. Also, Xia *et al.* [30] showed that, curcumin supplementation has significantly improved the growth of juvenile M. amblycephala. In addition, black pepper enhanced the absorption of curcumin, through increasing the blood concentration that extended the absorption and bioavailability of curcumin [8]. Moreover, azolla has the ability to fix the atmospheric nitrogen and removal the accumulation of mercury, arsenic, cadmium, lead, chromium, platinum and gold from waters [31]. So, it can remove the stress of pollutants that affected fish and give the chance to fish for growing normally. The current study revealed a significant increase in the activities of both GHR and IGF genes in liver tissue of *Oreochromis niloticus* that was exposure to azolla. These results are similar to those were recorded by other researchers [32].

There was a significant decrease in the activities of both GHR and IGF genes in the liver tissue of *Oreochromis niloticus* that were collected from waste water that was attributed to the increase of heavy metals in waste water that lead to decrease both of GHR and IGF. These results are in agreement with others [33] who reported decrease in both of GHR and IGF in rainbow trout (*Oncorhynchus mykiss*) after it was exposed to cobalt and zinc.

Conclusion

It can be concluded that addition of turmeric and black pepper powder to fish diet resulted in a significant increase in fish weight as well as an improvement in both of growth

hormone receptor gene and insulin like growth factor gene activities in liver tissue of Nile tilapia fish *(Oreochromis niloticus)* that were collected from waste water. Also, Azolla has the ability to improve fish weight as well as both of growth hormone receptor gene and insulin like growth factor gene activities in liver tissue of Nile tilapia fish in waste water.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

تأثير الكركم والفلفل الأسمر والأزولا علي أسماك البلطي النيلي المستزرعه علي المياه الملوثه

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[٢] المعمل المركزي لبحوث الثروة السمكية – العباسة

الهدف من هذا البحث هو معرفة مدي تأثير مادة الكركم مضافا اليه مسحوق الفلفل الأسمر علي وزن سمكة البلطي التي تم إستزراعها في المياه الملوثة كذلك تأثير الكركم مع الفلفل الأسمر علي التعبير الجيني لهرمون النمو والأنسولين كعامل مؤثر علي النمو كذلك معرفة تأثير نبات الأزولا في معالجة الأسماك المستزرعه في المياه الملوثة. تم التجربة علي ٢٥٠ سمكه بلطي نيلي وتم تقسيمهم إلي ٥ مجموعات تشمل كل مجموعة علي ٥٠ سمكة: المجموعه الاولي تم إستزراعها بأحواض صحيه بالمعمل المركزي لبحوث الثروه السمكيه بالعباسه وتستخدم كمجموعة ضابطة للتجربه. المجموعه الثانيه تم تجميعها من المياة الملوثة (منطقة بحر البقر) والتي تم تغذيتها بدون أي إضافات. المجموعه الثالثه تم تجميعها من المياه الملوثه والغير صالحه للإستخدام الأدمي (منطقة بحر البقر) ثم تم معالجتها بإضافة مسحوق الكركم مع الفلفل الأسمر إلي الغذاء أثناء التجربه بنسبة ٠,٥٪ لكلا من الكركم والفلفل الأسمر لمدة ٤٥ يوم. المجموعه الرابعه جمعت من المياه الملوثه (منطقة بحر البقر) ثم تم معالجتها عن طريق وضع نبات الازولا الذي يحمل طحلب الانابينا الي حوض السمك طوال فترة التجربه (٤٥ يوما). المجموعه الخامسه جمعت من منطقة بحر البقرثم تم معالجتها بكلا من اضافة مسحوق الكركم مضافا إليه الفلفل الأسمر مع وضع نبات الأزولا الي حوض المعالجه خلال فترة التجربه. لوحظ أثناء التجربه زياده في وزن السمك في أول ١٥ يوم من بداية المعالجه علي المجموعه الأولي ولكن نقصان الوزن لوحظ علي الاسماك التي تم استزراعها في منطقة بحر البقر ولكن لوحظ ارتفاع الوزن علي المجموعه الثالثه ، الرابعه والخامسه بعد ٤٥ يوم من بداية المعالجه وكذلك لوحظ تحسن في كلا من هرمون النمو والأنسولين علي المجموعات التي تم معالجتها بإضافة مسحوق الكركم مع الفلفل الأسمر (المجموعه الثالثه) وكذلك علي المجموعه الرابعه التي تم معالجتها بالأزولا ولكن لوحظ تحسنا ملحوظا بالمجموعه الخامسه التي تم معالجتها بكلا من اضافة مسحوق الكركم مع الفلفل الأسمر إلي الغذاء مع وضع نبات الأزولا في حوض المعالجه.