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Effects of bergamot (*Citrus bergamia*) peel oil-supplemented diets on growth performance, haematology and serum biochemical parameters of Nile tilapia (*Oreochromis niloticus*)

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Abstract The present study investigated the effects of dietary *Citrus bergamia* peel oil (CBO) on growth performance, haematological and serum biochemical responses of Nile tilapia, *Oreochromis niloticus*. Volatile content of *C. bergamia* peel oil used in study was detected with GC/MS. The 99.47% of the analysed volatile components were identified in decreasing quantities as limonene, linalool and linalyl acetate. The experimental fish were divided into four groups stocked into triplicate tanks (20 fish/tank), and fed daily with an additive-free basal diet containing 0% (CBO_0), 0.5% ($CBO_{0.5}$), 1.0% (CBO_1), 2.0% (CBO_2) of bergamot peel oil for an 8-week period. Results showed that dietary bergamot peel oil especially at 0.5 g 100 g^{-1} significantly increased growth performance of fish ($p < 0.05$). The levels of haemoglobin and haematocrit significantly increased in fish fed with $CBO_{0.5}$ and CBO_1 compared with the CBO_0-fed group ($p < 0.05$), despite no significant difference was observed in CBO_2 group ($p > 0.05$). Highest haematocrit value was found in the $CBO_{0.5}$ group compared with the control group. The serum glucose of the fish fed with $CBO_{0.5}$ and CBO_1 feeds decreased significantly compared with the control group ($p < 0.05$). The highest total protein, lowest cholesterol and triglyceride in serum were observed in fish fed with bergamot oil–supplemented groups. According to the results, it was revealed that 0.5% bergamot oil supplementation to Nile tilapia diets optimizes the growth performance, feed utilization and health status related with blood parameters of the fish.

Keywords *Citrus bergamia* · Aquaculture · Essential oil · Blood parameters · Tilapia

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Introduction

In the present decade, there has been an increased interest in the use of herbal products in animal nutrition owing to the rising concern for use of synthetic products (Citarasu 2010). Plant-based feed additives are heterogeneous products extracted from different parts of plants such as leaves, bark, fruit, root, seed and peel (Bölükbasi et al. 2010; Marzoug et al. 2011; Kesbiç 2019). A number of studies have been conducted on the use of aromatic plants and products in livestock's feeds (Newbold et al. 2004; Benchaar et al. 2008). The oils extracted from plants and spices have been found to have different positive effects on feeding of livestock's especially in aquaculture (Acar et al. 2015). Previous studies have reported that herbal oils and their active compounds may enhance growth performance, feed utilization and improve immune response and animal welfare (Baba et al. 2016; Acar et al. 2018).

Bergamot (*Citrus bergamia*) has a greenish yellow shell colour and is a very acidic fruit. The commercial value of this fruit is derived from essential oils and volatile compounds found in these (Moufida and Marzouk 2003). Bergamot essential oil (BEO) is produced from *C. bergamia* fruit peel by cold pressing process and the oil is used in cosmetics and food industry (Mandalari et al. 2007). Like other citrus oils, BEO contains a high amount of volatile components. Limonene, linalyl acetate, linalool, ɣ-terpinene and β-pinene are the major volatile components of BEO and they carve out about 75–80% by weight of BEO (Furneri et al. 2012). Major compounds of BEO, limonene, linalyl acetate and linalool, are listed in Animal Drugs, Feeds, and Related Products (FDA 2017a) while ɣ-terpinene and β-pinene are given in Food for Human Consumption list (FDA 2017b) by U.S. Feed & Drug Administration.

Limonene is a highly readily available product for humans and animals that are readily absorbed by the digestive tract after oral administration (Igimi et al. 1974). The use of limonene strengthens the digestive system by regulating acidity so it can be protective for digestive system diseases (Van der logt et al. 2004). In addition, limonene reduces lipid peroxidation, prevents stress (Fukumoto et al. 2008) and damage from free radicals (Devi et al. 2004). Also, it regulates blood pressure (Kawakami et al. 2004) and improves effects of immune system (Del Toro-Arreola et al. 2005). Linalyl acetate is the major compound for bergamot oils and together with acetate ester of linalool both occur in conjunction (Hanneguelle et al. 1992). Linalyl acetate and linalool are found in many plant origin oils and these oils have been used as additives in animal feeds (Denli et al. 2004). Therefore, this study was designed to investigate the dietary effects of bergamot oil on the growth performance and blood parameters of the cultured fish species Nile tilapia (*Oreochromis niloticus*).

Materials and methods

Determination of volatile compounds of bergamot oil and diets

The bergamot oil used in the study was obtained from local market (Misbahçe, Balıkesir, Turkey) and diluted with high-purity ethanol at the ratio of 1/20, and the aromatic components were analysed using GC-MS (Shimadzu GCMS QP 2010 ULTRA). A capillary column (RTX-5MS; 30 m; 0.25 mm; 0.25 m) and helium were used as the carrying gas for the analysis. Temperatures of the column furnace, interface, ion source and the injection were set at 40 °C, 250 °C, 200 °C and 250 °C, respectively. The injection volume was 1 μL, and the split (1/5) method was utilized for the injection. During the analysis, 78-min furnace cycle was applied, comprising 3 min at 40 °C, 4 °C/min increase from 40 to 240 °C, 10 min at 240 °C, 4 °C/min increase from 240 to 260 °C, 10 min at 260 °C. The obtained chromatograms were compared with the W11N9 library and the components were identified.

Diet preparation and experimental design

Diets containing ≈ 35% protein and ≈ 10% fat were formulated at the Çanakkale Onsekiz Mart University Marine Science and Technology Faculty, Feed Laboratory. Ration and dry matter–based nutritional content of the experimental diets were presented in Table 1. Feed and feed raw materials samples were analysed for proximate composition according to AOAC (2003). Bergamot oil in quantities of 0% (CBO_0), 0.5% ($CBO_{0.5}$), 1% (CBO_1) and 2% (CBO_2), respectively, was added to the diets before pelleting of finished diets and their storage at − 20 °C until usage. Feeding experiment was performed in triplicate. For each repetition, clinically healthy 20 fish tank^{-1} were randomly weighed and separated. In total, 240 tilapias (2.57 ± 0.06 g) were used for the experiment. The fish were placed in the experimental system and fed 3 times a day with experimental diets until the demand of the feed was completed by fish, over 8 weeks. During the experiment fish were kept under 12L:12D photoperiod and the average parameters of water were recorded once a week with a multiprobe (YSI 556MPS) and throughout the experiment, the average temperature was 24.25 ± 0.4 °C, dissolved oxygen was 7.60 ± 0.30 mg L^{-1} and pH was 7.38 ± 0.16. After the feeding experiment was over, all fish were fasted 1 day, weighed again and blood was collected for analysis.

Table 1 Formulation of feed ingredients for the experimental diets

Components (%)	CBO_0	$CBO_{0.5}$	CBO_1	CBO_2
Fish meal[1]	25.00	25.00	25.00	25.00
Soybean meal[2]	32.00	32.00	32.00	32.00
Wheat flour	22.00	22.00	22.00	22.00
Corn starch	10.00	10.00	10.00	10.00
Vitamin and mineral mixture[1]	4.00	4.00	4.00	4.00
Fish oil[3]	7.00	6.50	6.00	5.00
Citrus bergamia oil	0.00	0.50	1.00	2.00
Nutritional contents (%)				
Protein	35.30	35.06	34.92	34.88
Fat	10.04	10.64	10.26	10.28
Ash	5.66	5.51	5.17	5.94
Volatile components (%) of CBO-supplemented diets				
β-Pinene	–	4.14	5.42	5.63
β-Myrcene	–	0.55	0.59	0.63
α-Terpinene	–	–	0.07	0.08
Para cymene	–	0.52	0.57	0.61
Limonene	–	17.18	28.02	34.31
γ-Terpinene	–	3.62	4.29	4.43
α-Terpinolene	–	0.16	0.26	0.21
Linalool	–	9.51	12.0	12.46
Linalyl acetate	–	28.36	32.52	38.01

[1] Anchovy fish meal. Koptur Balıkçılık, Trabzon, Turkey

[2] Soybean meal. Agromarin Yem San. ve Tic. A.Ş., İzmir, Turkey

[3] Anchovy fish oil. Agromarin Yem San. ve Tic. A.Ş,. İzmir, Turkey

Vitamin-mineral mixture: Vitamin A, 18000 IU kg^{-1}; Vitamin D_3, 2500 IU kg^{-1}; Vitamin E, 250 mg kg^{-1}; Vitamin K_3, 12 mg kg^{-1}; Vitamin B_1, 25 mg kg^{-1}; Vitamin B_2, 50 mg kg^{-1}; Vitamin B_3, 270 mg kg^{-1}; Vitamin B_6, 20 mg kg^{-1}; Vitamin B_{12}, 0,06 mg kg^{-1}; Vitamin C, 200 mg kg^{-1}; Folic acid, 10 mg kg^{-1}; Calcium d–pantotenat, 50 mg kg^{-1}; Biotin, 1 mg kg^{-1}; İnositol, 120 mg kg^{-1}; Colin chlorite, 2000 mg kg^{-1}; Fe, 75,3 mg; Cu, 12,2 mg; Mn, 206 mg; Zn, 85 mg; I, 3 mg; Se, 0,35 mg; Co, 1 mg

Calculation of growth performance and feed utilization parameters

Daily feed consumption (DFC), feed conservation ratio (FCR), specific growth rate (SGR) and relative growth rate (RGR) were calculated according to Yiğit et al. (2012).

Blood collection and analyses

At the end of the 8-week feeding experiment, 5 fish from each tank (15 fish per group) were anesthetized with clove oil and their blood was collected from the caudal vein by sterile injector. A portion of the sampled blood was collected to standard lavender-top blood collection tubes containing anticoagulant (EDTA) tubes for haematological analysis and the other part of blood put into standard red-top (SST™ II) advance serum separator tubes for extracted serum by centrifuge to be used in the biochemical parameters. Obtained sera were stored − 80 °C until analyses. Haematological analyses were performed rapidly; red blood cells (RBC) were counted under a light microscope. Cyanmethemoglobin method was used for determination of haemoglobin (HGB) concentration and haematocrit (HCT) value was observed by capillary HCT tube after centrifuge (Hesser 1960; Faggio et al. 2013; Fazio et al. 2014). Biochemical parameters such as serum glucose (GLU), total protein (TPROT), albumin (ALB), globulin (GLO), triglyceride (TRIG), cholesterol (CHO), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were determined in serum using bio analytic test kits (Bioanalytic Diagnostic Industry, Co.) and then measured with a spectrophotometer (Optizen POP UV/VIS).

Statistical analysis

One-way ANOVA was used for data analysis after checking the normality of the data and their variance homogeneity. When ANOVA was significant, multiple comparisons of the treatment mean values were assessed by the Tukey post-hoc test. Data were expressed as mean values ± SD. A difference in the mean values of $P < 0.05$ was considered to be significant. All statistical analyses and summary statistics were carried out using the SPSS statistical package version 16.0 (IBM, Armonk, NY, USA).

Results

Volatile compounds of *C. bergamia* oil

Detectable volatile components of *C. bergamia* oil are presented in Table 2.

Growth performance

The survival rate in all groups at the end of the 8 weeks feeding trial was 100%. Bergamot oil supplementation

Table 2 The aromatic content of the *Citrus bergamia* oil

Analyte	Retention time (s)	Concentration %
β-Pinene	3.611	6.79
β-Myrcene	3.673	0.96
α-Terpinene	4.229	0.10
Para cymene	4.387	0.43
Limonene	4.499	39.15
γ-Terpinene	5.158	5.46
α-Terpinolene	5.943	0.23
Linalool	6.184	16.45
Linalyl acetate	11.276	29.90
	Total	99.47

of tilapia feed significantly affected growth performance and feed utilization of tilapias ($p < 0.05$) (Table 3).

Haematology parameters

Dietary supplementation of bergamot oil significantly affected red blood cell count (RBC), haemoglobin value (HGB) and haematocrit percentage (HCT) ($p < 0.05$) (Table 4). The rates of HGB and HCT significantly increased in fed with $CBO_{0.5}$ and CBO_1 groups tilapias compared with the CBO_0 (control) ($p < 0.05$), in spite of no significant difference observed in CBO_2 group ($p > 0.05$). Highest HCT value was observed in the $CBO_{0.5}$ group compared with the control group.

Blood biochemical parameters

The results of blood biochemical parameters of tilapias after 8 weeks of feeding on experimental feeds supplemented *Citrus bergamia* oil are shown in Table 5. The serum glucose of the fish fed with $CBO_{0.5}$ and CBO_1 feeds decreased significantly compared with the control group ($p < 0.05$). The highest total protein, lowest cholesterol and triglyceride in serum were observed in fish fed with bergamot oil–supplemented groups. Though, there were no significant differences in GOT and AL*P* values ($p > 0.05$), GPT and LDH values of liver enzymes showed significant differences between groups ($p < 0.05$).

Discussion

The use of plant-delivered oils in fish feeds have shown several positive effects on fish such for example anti-stress, growth booster, digestive-regulating, immunostimulating and antibacterial (Citarasu 2010). Different types of bergamot extract have a long medicinal history (Navarra et al. 2015) and they are highly characterized by limonene, linalool and linalyl acetate (Han et al. 2017) as used in the present study. Some aromatic plants and extracts have been reported to increase feed consumption of the growth stimulating effect when used as a feed additive. (Santoso et al. 2013). Any previous study on the effect of *Citrus bergamia* oil on growth performance, health status parameters or investigating dose trials to determine effective feed intake of *O. niloticus* feeding are known in the literature. Recent studies suggested that other citrus species are effective on these parameters in fish and most of them have reported positive effects on growth performance and health parameters of fish for the oil extracted from the citrus peels (Acar et al. 2015; Baba et al. 2016). The oil extracted from the citrus species peels contain a large amount of limonene (Song et al. 2000; Asikin et al.

Table 3 Growth performance *Tilapia niloticus* fed *Citrus bergamia* oil supplemented diets after 60 days

	Treatment				SEM*	*p* value
	CBO_0	$CBO_{0.5}$	CBO_1	CBO_2		
Initial weight (g)	2.60 ± 0.06	2.58 ± 0.05	2.52 ± 0.08	2.60 ± 0.04	0.018	0.343
Final weight (g)	9.39 ± 0.19^b	11.61 ± 0.28^a	9.90 ± 0.36^b	9.64 ± 0.38^b	0.274	< 0.001
FCR	1.30 ± 0.02^a	0.91 ± 0.02^c	1.06 ± 0.05^b	1.29 ± 0.10^a	0.051	< 0.001
RGR (%)	261.43 ± 4.22^b	350.31 ± 11.77^a	293.7 ± 25.7^b	270.06 ± 10.29^b	11.099	< 0.001
SGR (%/day)	5.21 ± 0.09^b	5.75 ± 0.11^a	5.52 ± 0.27^{ab}	5.26 ± 0.04^b	0.075	0.009

In the same line, values (mean ±SD) of three replicate tanks. With different superscripts are significantly different ($p < 0.05$). *DFI*, daily feed intake; *FCR*, feed conversion rate; *RGR*, relative growth rate; *SGR*, specific growth rate. *Standard error of mean value

Table 4 Haematology parameters in *Tilapia niloticus* fed *Citrus bergamia* oil after 60 days

	Treatment				SEM*	*p* value
	CBO_0	$CBO_{0\cdot5}$	CBO_1	CBO_2		
RBC (10^6cell/mm^3)	1.92 ± 0.08^b	2.84 ± 0.35^a	2.96 ± 0.38^a	2.19 ± 0.30^b	0.068	< 0.001
HGB (g/dL)	6.87 ± 0.49^b	9.18 ± 0.56^a	8.95 ± 0.50^a	7.07 ± 0.76^b	0.155	< 0.001
HCT (%)	31.56 ± 3.19^b	36.56 ± 4.94^a	34.59 ± 2.78^{ab}	34.15 ± 1.81^{ab}	0.484	0.002

In the same line, values (mean ± SD) of 15 fishes per treatment. With different superscripts are significantly different ($p < 0.05$). RBC, red blood cell count; *HGB*, haemoglobin concentration; *HCT*, haematocrit rate; *Standard error of mean value

2012; Acar et al. 2015; Baba et al. 2016). The oil used in this study contained 39% limonene and it was observed an increasing of the growth performance and feed utilization rates of the fish fed with experimental feeds. Similar results have been reported in a study of tilapia feeds supplemented with pure limonene. In addition, it was reported that *Igf-I* gene expression levels increased on fish fed with limonene addicted feed compared with control (non-limonene) (Aanyu et al. 2018).

The effect of herbal supplements as growth promoters, when included as fish diet additives, presumably acts on the intestinal microbiota, which can have direct or indirect effects on growth (MacLennan et al., 2002). The gut-secretion-stimulating effect of essential oils also allows the microbiota to modulate and improve digestion and the absorption of nutrients. This stimulation may provide a greater array of amino acids for protein synthesis and thus increase body protein content (Freccia et al., 2014). These results also indicate the importance of suitable dosing to obtain the desired effects (Reverter et al., 2014). There are many positive aspects of medicinal plants in fish, but there are also side effects from active component and overdose. However, they do not cause any problems at the appropriate dosage (Ahmad et al. 2006).

In recent years, haematology is an important diagnostic tool that can be used as an effective and sensitive index to monitor the feed changes in fishes (Parrino et al. 2018). Haematological parameters are an important guide for assessing the health of fish, as it serves as a reliable indicator of their physiological state which is of significant importance to aquaculture industry (Fazio 2018; Harikrishnan et al. 2010). Previous studies reported that the haematological parameters of unhealthy fish decrease (Chen et al. 2004). Conversely, it has been reported that plant sources containing volatile components increase haematological parameters in fish (Farahi et al. 2010; Fallahpour et al. 2015). In the present study, RBC, HGB and HCT values of fish fed with supplemented feeds have significantly increased ($p < 0.05$).

Table 5 Blood biochemical parameters of *Tiapia niloticus* fed different levels of *Citrus bergamia* oil–supplemented diets

	Treatment				SEM*	*p* value
	CBO_0	$CBO_{0.5}$	CBO_1	CBO_2		
GLU (mg dL^{-1})	91.41 ± 3.49^a	61.34 ± 5.54^c	68.26 ± 7.85^b	87.91 ± 7.24^a	1.835	< 0.001
TPROT (g dL^{-1})	3.45 ± 0.40^b	4.72 ± 0.76^a	4.87 ± 0.67^a	4.42 ± 0.81^a	0.112	< 0.001
ALB (g dL^{-1})	1.44 ± 0.26	1.31 ± 0.19	1.27 ± 0.16	1.41 ± 0.19	0.027	0.08
CHOL (mg dL^{-1})	100.24 ± 10.14^a	74.88 ± 9.83^b	73.21 ± 5.85^b	71.96 ± 9.33^b	1.893	< 0.001
TRIG (mg dL^{-1})	87.83 ± 8.10^a	73.59 ± 8.39^b	72.22 ± 9.96^b	74.24 ± 6.14^b	1.325	< 0.001
GOT (U L^{-1})	103.94 ± 11.27	98.44 ± 9.63	99.36 ± 12.06	108.33 ± 11.44	1.513	0.073
GPT (U L^{-1})	23.98 ± 3.64^a	19.32 ± 2.17^{bc}	18.08 ± 1.91^c	20.56 ± 1.72^b	0.423	< 0.001
ALP (U L^{-1})	71.99 ± 5.14	71.11 ± 4.45	72.03 ± 5.34	70.80 ± 4.08	0.605	0.059
LDH (U L^{-1})	224.28 ± 17.98^a	203.99 ± 15.67^{bc}	189.31 ± 19.54^c	209.31 ± 22.02^{ab}	2.886	< 0.001

In the same line, values (mean ± SD) of 15 fishes per treatment. With different superscripts are significantly different ($p < 0.05$). *GLU*, glucose; *TPROT*, total protein; *ALB*, albumin; *CHOL*, cholesterol; *TRIG*, triglycerides; *GOT*; glutamic-oxaloacetic transaminase; *GPT*, glutamic-pyruvic transaminase; *ALP*, alkaline phosphatase; *ALP*, lactate dehydrogenase. *Standard error of mean value

Similar results have been observed in studies about tilapia feeding with oils obtained from different citrus species peels (Acar et al. 2015).

Serum biochemical changes that occur in fish blood have been often used as reference parameters to understand the effects of fish feed ingredients, fish metabolism and health (Yilmaz et al. 2018). Especially serum GLU level is one of the important stress indicators detectable in serum. Other studies have reported an augment in serum GLU level with the increasing stress in aquaculture application (Silbergeld 1974). In the present study, the serum GLU levels of the fish fed with CBO_2 group were shown no significant differences ($p > 0.05$) while the GLU values of the fish fed with $CBO_{0.5}$ and CBO_1 significantly decreased compared with the control group ($p < 0.05$). The major compound of *Citrus bergamia* oil, d-limonene has been designated as a chemical compose with low toxicity based upon lethal dose (LD50) (Kim et al. 2013) besides. In addition to this, sedative (anxiolytic and antidepressant) effects of the oils obtained from citrus species have also been reported (Komiya et al. 2006). Similar results have been obtained in the supplementation of sweet orange (Acar et al. 2015) and lemon peel essential oil (Baba et al. 2016) in *Oreochromis mossambicus*. Serum proteins amount have been an indicative value of unhealthy conditions related to diet for fish (Bernet et al. 2001). Due to the serum proteins carry the materials required for metabolism such as free fatty acids and Fe, the increase in plasma implies that immunity in fish has been enhanced (Rankin 1992). Serum TPROT level was significantly increased ($p < 0.05$) in fish fed with all CBO supplement feed compared with the control (non CBO), in the case of ALB value, there were no differences among all groups in the current study. Previous studies obtained similar results in fish fed with different plant-based oils (Awad et al. 2013; Acar et al. 2015; Baba et al. 2016). Limonene, the main component of bergamot oil, has a hypolipidemic effect. Previous researchers have reported that consumption of limonene-containing fats in diets reduces the serum total cholesterol, low-density lipoprotein (LDL, bad cholesterol) and triglyceride, and increase high-density lipoprotein (HDL, good cholesterol) (Vieira et al. 2018; Ahmad and Beg 2013). The results of the present study, in line with previous studies on supplementation of lemon (Baba et al. 2016) and orange peel oil (Acar et al. 2015) in tilapia feeds, supported the hypolipidemic effect of citrus oil. According to previous studies on serum liver enzymes as aminotransferase (GOT and GPT), ALP and LDH increase, have been indicated as markers for a negative impact on liver health (Gliozzi et al. 2014). In current study, it was observed that fish's GPT and LDH values significantly decreased in all experimental groups compared with the control group, whereas any significant differences have been marked considering the ALP and GOT values. This is thought to be due to two different reasons. First, increased blood lipid levels such as cholesterol and triglycerides in the metabolism may disrupt liver health (Gliozzi et al. 2014). Second, limonene found in bergamot oil has a high antioxidant capacity (De Almeida et al. 2014), so it can increase liver health by preserving the membrane of hepatic tissue (Gliozzi et al. 2014).

Conclusion

Bergamot (*Citrus bergamia*) is not a widely consumed form of fruit but the oil extracted from the peel of bergamot contains limonene in high amounts. The growth performance, haematology values and blood biochemistry parameters of Nile tilapia (*O. niloticus*) fed with experimental diets was improved. According to the results, it can be concluded that 0.5% bergamot oil supplementation in Nile tilapia diets optimizes growth performance, feed utilization and health status agreed with blood parameters.

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