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Effects of several macerated citrus oils on health parameters of rainbow trout (*Oncorhynchus mykiss*) under high stocking density

Efectos de varios aceites cítricos macerados sobre los parámetros de salud de la trucha arco iris (*Oncorhynchus mykiss*) bajo alta densidad de población

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ABSTRACT

The interplay between dietary components and antioxidant systems in fish physiology is crucial for aquaculture. Citrus fruits, renowned for their high content of bioactive antioxidants, have attracted attention for their antioxidant features in fish. The purpose of this study was to investigate the dietary effects of using essential oil from macerated oils of lemon peel (*Citrus limon*) (MOL), orange peel (*Citrus sinensis*) (MOO) and grapefruit peel (*Citrus paradisi*) (MOG) on growth, proximate composition, hematological, antioxidant parameters of rainbow trout (*Oncorhynchus mykiss*) subjected to high stocking density stress. Fish (110 ± 10.95 g) were randomly introduced to three trial groups (1% of MOL, MOO and MOG). At the end of feeding period (42-day), the results showed that the three trial groups had significant effects on the feed conversion ratio (FCR), especially in the MOG (P<0.05). The FCR value in MOG was remarkably 0.81. In the study, the lowest dry matter and ash levels were detected in MOO, moisture content in MOL, but the highest fat (6.82 ± 0.82) and protein (15.85 ± 0.74) levels were found in MOG. There were significant differences in proximate composition and red blood cell (RBC) (1.73 ± 0.07 in MOG), hemoglobin (Hgb) (8.97 ± 0.43 in MOL), hematocrit (Hct) (23.92 ± 1.09 in MOL), mean corpuscular hemoglobin concentration (MCHC) (38.22 ± 0.34 in MOG) between the control and all groups (P<0.05). The results showed that glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR) activities in all treatment groups were decreased and malondialdehyde (MDA) level was increased in the MOO group activity (P>0.05).

Key words: stress on fish; herbal oils; food supplements; fish physiology

RESUMEN

La interacción entre los componentes de la dieta y los sistemas antioxidantes en la fisiología de los peces es crucial para la acuicultura. Los cítricos, conocidos por su alto contenido en antioxidantes bioactivos, han llamado la atención por sus características antioxidantes en los peces. El propósito de este estudio fue investigar los efectos dietéticos del uso de aceites esenciales de aceites macerados de cáscara de limón (*Citrus limon*) (MOL), cáscara de naranja (*Citrus sinensis*) (MOO) y cáscara de pomelo o toronja (*Citrus paradisi*) (MOG) sobre el crecimiento, composición próximal, parámetros hematológicos y antioxidantes de la trucha arcoíris (*Oncorhynchus mykiss*) sometida a estrés por alta densidad. Para el estudio se introdujeron aleatoriamente peces (110 ± 10,95 g) en tres grupos de prueba (1 % de MOL, MOO y MOG). Al final del período de alimentación (42 días), los resultados mostraron que los tres grupos de prueba tuvieron efectos significativos en el factor de conversión alimentcia (FCR), especialmente en el MOG (P<0,05). El valor del FCR en MOG fue notablemente de 0,81. En el estudio, los niveles más bajos de materia seca y cenizas se detectaron en MOO, el contenido de humedad en MOL, pero los niveles más altos de grasa (6,82 ± 0,82) y proteína (15,85 ± 0,74) se encontraron en MOG. Hubo diferencias significativas en la composición proximal y en los glóbulos rojos (RBC) (1,73 ± 0,07 en MOG), hemoglobina (Hgb) (8,97 ± 0,43 en MOL), hematocrito (Hct) (23,92 ± 1,09 en MOL), concentración corpuscular media de hemoglobina. (MCHC) (38,22 ± 0,34 en MOG) entre el control y todos los grupos (P<0,05). Los resultados mostraron que las actividades de la glutatión peroxidasa (GPx), catalasa (CAT) y glutatión reductasa (GR) en todos los grupos de tratamiento disminuyeron y el nivel de malondialdehído (MDA) aumentó en la actividad del grupo MOO (P>0.05).

Palabras clave: estrés sobre el pescado; aceites vegetales; complementos alimenticios; fisiología del pez

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INTRODUCTION

Citrus fruits, including oranges (*Citrus sinensis*), lemons (*Citrus limon*), limes, and grapefruits (*Citrus paradisi*), are renowned for their rich content of bioactive compounds such as flavonoids, phenolic acids, and vitamin C. Additionally, citrus flavonoids, such as hesperidin and naringin, have been shown to possess anti-inflammatory, antimicrobial, and antiviral properties [1]. These compounds exhibit potent antioxidant properties, which have been linked to various health benefits, including protection against oxidative stress-related diseases.

Furthermore, citrus essential oils, extracted from the peels or leaves of citrus plants, contain a myriad of volatile compounds with documented antimicrobial and immunomodulatory effects. These natural compounds have demonstrated a sustainable alternative to conventional chemotherapeutic agents and antibiotics [2]. There has been a growing interest in exploring natural remedies for promoting fish antioxidant system and enhancing aquaculture practices. Citrus species have emerged as a promising avenue due to their rich phytochemical ingredients and diverse biological activities [3].

The potential health benefits of citrus species on fish have garnered attention for their application in aquaculture. Studies have suggested that incorporating citrus extracts or by-products for examples oils or pulp etc. into fish diets can confer various physiological and immunological advantages, ultimately improving growth performance, antioxidant capacity, and overall well-being [4].

Citrus species can aid in combating infectious diseases commonly encountered in aquaculture settings. Despite the promising findings from experimental studies, there remains a need for comprehensive reviews that synthesize the existing literature and provide insight into the mechanisms underlying the health-promoting effects of citrus species in fish [5]. Macerated oils are oils obtained by keeping certain parts of plants in carrier oils such as sunflower or olive oil for 15-20 days (d). Macerated oils contain both therapeutic and nutritional properties. In the extraction method, only small molecules are transferred to the oil, while in the macerated method, large molecules are transferred to the oil. Thus, it becomes a complex consisting of the majority of the fat-soluble therapeutically effective substances in the plant. For these reasons, macerated oils are seen as a whole [6].

Essential oils and pressed oils were generally used as food additives in aquaculture. Studies on macerated oils were quite limited. In this study, the aim to provide a comprehensive overview of the current state of knowledge regarding the benefits of citrus species on fish health (7, 8). Through an analysis of relevant research articles, macerated citrus oils will explored the antioxidant and hematologic parameters of fish as well as their potential applications in aquafeed formulations. By consolidating this information, we hope to stimulate further research efforts and facilitate the integration of citrus-derived products into sustainable aquaculture practices.

MATERIALS AND METHODS

Fish material and experimental design

The investigation took place within the fisheries Department of Malatya Turgut Ozal University. macerated oils of lemon peel (MOL), macerated oils of orange peel (MOO), and macerated oils of grapefruit peel (MOG) oils were generated utilizing the maceration technique. After the dried peels of Citrus species were crushed in a grinder (EMIR, YB-30 BL, Turkey), they were kept in glass jars at 100 g·L^{-1} for 15 d. At the end of the waiting period, it was filtered with filter paper and stored in lightproof bottles and kept in a cool place. The macerated oils were incorporated into trout feed at a rate of 1%. The experiment was executed following a completely randomized design comprising five treatments, each replicated twice, control group (normal, unstress): 10 fish, control stress group (high density): 50 fish, three citrus groups (high density): 50, totaling 210 fish for each replicationwere used. The average weight (TEM scales, Ns6200, Türkiye) of rainbow trout in the experiment was 110 ± 10.95 g. Throughout the trial, fish were fed with feed equal to 2% of their total body weight twice daily for 42 d. Key water parameters, including average temperature (8.3 ± 0,1°C), conductivity (16.2 ± 0,01 µS·cm^{-1}), hardness (10.52 ± 0,01 mg·L^{-1}), salinity (8.6 ± 0,1 ppt o g·L^{-1}), pH (8.2 ± 0,2), and dissolved oxygen (7.49 ± 0,02 mg·L^{-1}), were assessedusing pH meter with multiprobe (EZDO – Waterproof Handheld pH Tester 6011, China) during the experiment.

Hematologic and antioxidant analysis

Upon completion of the experiment, blood was sampled from the caudal vein of individual fish subsequent to their sedation with benzocaine (25 mg·L^{-1} water) [9]. Blood samples were procured from the tail veins of the anesthetized fish and transferred to ethylenediaminetetraacetic acid (EDTA) containing tubes. Hematological analysis was conducted using the Fully Auto Hematology Analyzer (PROCAN PE-6800VET, China). The blood samples were refrigerated for one day at 4°C. For plasma extraction, samples underwent centrifugation at 1000 G for 15 min. Glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) activities and malondialdehyde (MDA) level were assessed in plasma using commercial kits (SunLong Biotech Co., LTD, China). Plasma homogenates were analyzed following kit instructions, and absorbance values were measured at 450 nm using a microplate reader (DR-200Bc Microplate Reader, Prokan Electronics, China, Shanghai YL). The results were computed as per the provided instructions.

Growth parameters

The parameters assessing growth performance, including weight gain (WG) percentage, specific growth rate (SGR), and feed conversion ratio (FCR), were computed following the methodology outlined by [10].

The formula for weight gain (%) is:

$$Weight\ gain\,(\%) = \left(\frac{Final\ weight - Initial\ weight}{Initial\ weight}\right) \times 100$$

SGR is calculated as:

$$SGR = \left(\frac{Final\ weight - Initial\ weight}{days}\right) \times 100$$

FCR is determined by dividing the feed offered by the weight gain in grams. The determination of the FCR occurred at the conclusion of the 42-d feeding period, with each fish in the respective tanks individually weighed, and the FCR calculated as per the method described by Gultepe in 2018 [11].

$$FCR = \frac{Total\ Feed\ Consumed}{Total\ Weight\ Gain}$$

Proximate composition

The composition of fish muscle, in terms of dry matter, moisture, fat, total proteins and ash of the muscle samples of fish was analyzed following the procedure outlined in reference [12]. Moisture was assessed using a gravimetric technique, in which the sample was dried at 105°C until it achieved a constant weight. Crude protein levels were determined using the Micro Kjeldahl method (6.2 × N) (EFLAB, MGD1000X, Türkiye). Total lipid content was quantified by extracting (Soxhlet system) with light petroleum ether, with subsequent removal of the solvent through distillation. Ash content was determined from the residue remaining after incineration in a muffle furnace at 550°C for approximately 20 hours.

Statistical analyses

Prior to analysis, data underwent normalization using the Kolmogorov–Smirnov method and were subsequently subjected to two-way analysis of variance (ANOVA). Mean comparisons were conducted using Duncan's statistical test at a significance level of 95%. Statistical analyses were performed using SPSS 25 software, while graphical representations were created using Excel 2016 software, as referenced [13, 14, 15].

RESULTS AND DISCUSSION

Growth indices

Although the highest weight gain among the groups and the SGR were highest in the control group, FCR values were strikingly more efficient in the citrus groups according to control groups. FCR in treatments containing MOG showed the most significant difference in comparison with control treatment after exposure to MOG. In addition, second and third significant differences in FCR values were observed in MOL and MOO groups (TABLE 1).

TABLE I
Growth performance of rainbow trout fed diets containing different macerated citrus oils concentrations for 42 days

Variable	Groups				
	Control	Control stress	MOL	MOO	MOG
IW	122±1.45	107.5± 1.15	107.0 ±1.05	106.5±1.15	107.5±1.10
FW	228±3.10	188.8±2.70	201.1±2.90	164.0±2.15	190.4±2.40
WG	106±3.10	81.3±2.30	94.1±2.10	57.5±1.00	82.9±1.35
FCR	1.16±0.13	1.12±0.10	0.93±0.08	1.04±0.11	0.81±0.06
SGR	2.71±0.09	2.08±0.12	2.41± 0.09	1.47±0.13	2.12±0.08

IW: initial weight, FW: final weight, WG: weight gain, FCR: feed conversion ratio, SGR: specific growth rate is expressed in % per day^{-1}, MOL: macerated oil of lemon peel, MOP: macerated oil of orange peel, MOG: macerated oil of grapefruit peel

Proximate muscle analysis

Statistical differences were detected in dry matter, moisture, ash, protein levels except lipid level in the application groups (TABLE II). Muscle dry matter content of fish in treatment containing MOO decreased significantly compared to other groups (P<0.05). The amount of ash in the MOO group was decreased among three treatments (P<0.05). The fat level in muscle was not significantly different treatments (P>0.05).The amounts of muscle protein declined significantly in MOG group compared to the control (P<0.05).

TABLE II
Proximate composition of the musculature of control and experimental groups (Mean ± Standard Deviation)

Variable	Groups				
	Control	Control stress	MOL	MOO	MOG
Dry matter	24.28±0.35^{a}	24.71±0.25^{a}	24.99±0.12^{a}	23.14±0.19^{b}	24.19±0.80^{ab}
Moisture	75.71±0.54^{ab}	75.29±0.21^{a}	75.05±0.21^{a}	76.86±0.37^{bc}	75.80±0.43^{ac}
Ash	1.16±0.15^{a}	1.55±0.11^{ab}	1.49±0.05^{a}	1.09±0.35^{a}	1.26±0.12^{a}
Fat	6.26±0.75^{a}	6.45±1.02^{a}	6.82±0.82^{a}	6.59±0.69^{a}	6.18±0.56^{a}
Protein	15.76±0.57^{a}	15.82±0.67^{ab}	15.85±0.74^{ab}	14.45±0.44^{ab}	15.71±0.36^{b}

MOL: macerated oil of lemon peel, MOP: macerated oil of orange peel, MOG: macerated oil of grapefruit peel

Blood and serum parameters

According to the blood parameters data in TABLE III; red blood cell (RBC), hemoglobin (Hgb), hematocrit (Hct), mean corpuscular hemoglobin concentration (MCHC) counts significantly dropped in all treatments (P<0.05). But white blood cell (WBC) count decreased and lymphocyte (LYM), monocyte (MID), granulocyte (GRAN) levels elevated considerably in all treatments groups compared to the control stress group.

TABLE III
Hematological parameters (Mean ± Standard Deviation)

Parameters	Groups				
	Control	Control stress	MOL	MOO	MOG
WBC (10^{3}·µL^{-1})	47.80±5.24^{a}	56.37±1.26^{a}	52.50±2.62^{a}	53.43±0.90^{a}	53.12±2.01^{a}
LYM (%)	94.02±0.21^{a}	84.93±9.03^{a}	93.12±0.30^{a}	93.28±0.55^{a}	93.37±0.45^{a}
MID (%)	3.98±0.24^{a}	4.10±0.19^{a}	4.43±0.18^{a}	4.38±0.36^{a}	4.45±0.28^{a}
GRAN (%)	2.00±0.09^{a}	1.97±0.11^{a}	2.45±0.14^{a}	2.33±0.20^{a}	2.18±0.07^{a}
RBC (10^{6}·µL^{-1})	1.53±0.18^{a}	1.87±0.07^{b}	1.72±0.08^{ab}	1.69±0.04^{ab}	1.73±0.07^{ab}
Hgb (g·dl^{-1})	7.90±0.94^{a}	9.72±0.36^{b}	8.97±0.43^{ab}	8.72±0.12^{ab}	8.90±0.48^{ab}
Hct (%)	21.32±2.46^{a}	26.03±0.88^{b}	23.92±1.09^{ab}	23.28±0.33^{ab}	23.23±1.14^{ab}
MCV (fL)	139.43±1.52^{a}	139.35±1.42^{a}	139.55±1.18^{a}	138.32±1.27^{ab}	134.65±1.25^{b}
MCH (pg)	51.35±1.11^{a}	51.85±0.91^{a}	52.17±0.87^{a}	51.65±0.77^{a}	51.38±0.71^{a}
MCHC (g·dl^{-1})	36.87±0.44^{a}	37.25±0.36^{ab}	37.43±0.39^{ab}	37.38±0.34^{ab}	38.22±0.34^{b}

MOL: macerated oil of lemon peel, MOP: macerated oil of orange peel, MOG: macerated oil of grapefruit peel, WBC: white blood cell, LYM: lymphocyte, MID: monocyte, GRAN: granulocyte, RBC: red blood cell, Hgb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration

Antioxidant parameters

MOO treatment alone caused significant increase in the GPX activity of the plasma (P<0.05). All treated groups showed no significant change in the GR and CAT activities compared with the control (P>0.05). It was found that the MDA activities were higher compared with the control group in all examples (P<0.05, FIGURE 1).

Optimizing feed efficiency is a critical aspect of aquaculture management, directly impacting production costs and environmental

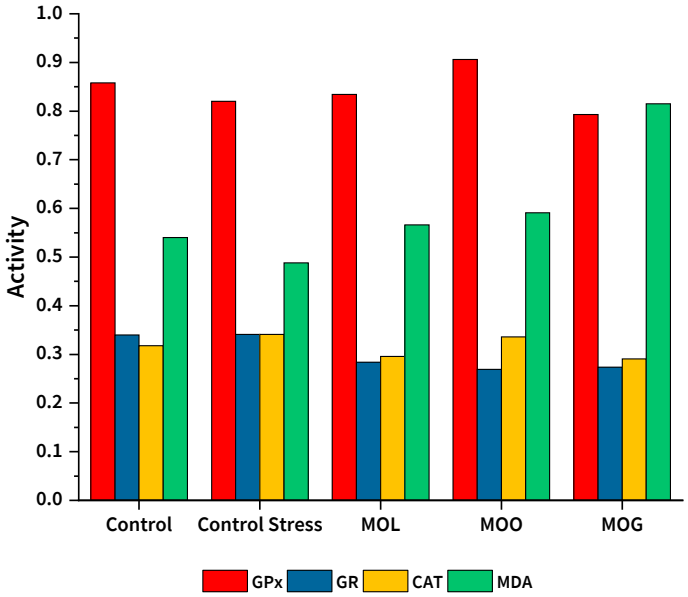

FIGURE 1. The values of GPx, GR, CAT activities and MDA level of experimental groups (Mean ± Standard Deviation). MOL: macerated oil of lemon peel, MOP: macerated oil of orange peel, MOG: macerated oil of grapefruit peel, GPx: glutathione peroxidase, GR: glutathione reductase, CAT: catalase, MDA: malondialdehyde

sustainability. Citrus fruits, renowned for their rich nutritional profile and bioactive compounds, have been explored as potential dietary supplements to improve fish performance and feed utilization efficiency. Several studies have been conducted recently to assess the impact of citrus products on the growth performance of several fish species. They found that WG, FCR and SGR values increased in rainbow trout fed with feed supplemented with *Ferulago angulata* extract, similar to our study [16].In another study conducted on rainbow trout of mavigreek, the best FCR value was found in the control group, while in our study, MOG (0.81) < MOL (0.93) < MOO (1.04) < control (1.12) values gave better results than the control group [17]. Supplementing diets with lemon and orange essential oils enhanced growth performance of tilapia [18]. In the same context, the addition of grapefruit (*Citrus paradise*) peel extract to caspian white fish (*Rutilus kutum*) diets increased fish growth performance [19]. Adel *et al.* investigated the impact of dietary supplementation with lemon verbena (LV) (*Aloysia citrodora*) extract on growth performance and feed efficiency in Siberian sturgeon (*Acipenser baerii*). They reported a significant improvement in the growth performance and feed utilization [20].

Ebtehal *et al.*, reported a significant increase in protein content and a decrease in lipid content in fish fed with citrus peel meal compared to the control group. The authors attributed this improvement to the high fiber content and bioactive compounds present in citrus peels, which positively influenced nutrient digestibility and utilization in fish [21].This is similar to the positive results on the FCR value of citrus oils.

Resketi *et al.* [22] examined the influence of dietary supplementation with citrus extracts on the proximate composition of rainbow trout. They observed a significant increase in the protein content and a decrease in lipid content in fish fed with citrus extracts compared to the control group; in that study, there was no statistically significant effect of the citrus oils given on the proximate analysis.

It has been observed that blood production in fish is stimulated as a result of stress due to intensive stocking. In our study, it was observed that citrus macerated oils suppressed blood production caused by stress in RBC, Hgb, Hct and MCHC values. This suggested that this situation provided a negative feedback. Similar to this findings, extracts of orange and lemon had no marked effects on the haematological indices including Hgb and RBC of the cultured fish [18].

In another study, orange essential oil and lemon essential oil has not affected on the haematological indices including Hb and RBC of the cultured fish [23]. In a study conducted on rainbow trout using macerated tomato and carrot oils, significant differences were detected in MCH, RBC, HGB, HCT, LYM values [6]

Vicente *et al.* found that RBC, Hgb, Hct and MCHC values did not change in Nile tilapia when orange peel was applied [24]. Sgarro *et al.* [25] investigated the impact of dietary supplementation with orange and lemon extracts on Nile tilapia. They observed a significant increase in RBC count, Hgb concentration, and Hct levels in fish fed with citrus extracts compared to the control group. Our similar results were attributed to the antioxidant properties of citrus bioactive compounds, which potentially enhance erythropoiesis and oxygen-carrying capacity in fish.

In an another trial on hematologic parameters in rainbow trout, Macedo *et al.* investigated the effects of dietary supplementation with citrus peel extract. They reported a significant increase in WBC and LYM levels in fish fed with citrus peel extract compared to the control group. In our application, it was suggested that the immunomodulatory properties of citrus bioactive compounds might enhance the immune response of fish, leading to alterations in hematologic profiles [26].

Most applications on citrus species involved peels, essential oils, and extracts. In our study conducted in plasma, a lower or no antioxidant effect was observed. However, the increase in GPx values in the MOL and the MOO groups attracted attention.

The interplay between dietary components and antioxidant systems in fish physiology is crucial for maintaining cellular homeostasis and mitigating oxidative stress. Citrus fruits, renowned for their high content of antioxidants, have attracted attention for their potential to modulate antioxidant parameters in fish.

In the study conducted by Altinterim [27] in rainbow trout with macerated sesame oil, an increase in GPx level was detected, similar to that in our macerated lemon group. Chekani *et al.* [28] investigated the influence of dietary supplementation with citrus peel extract on blood antioxidant parameters in African catfish. They reported a significant increase in total antioxidant capacity (TAC) and GPx activity in the blood of fish fed with citrus peel extract compared to the control group. Similar to present study, increasing GPx level by bioactive compounds from citrus enhanced the fish's antioxidant defense system, thereby reducing oxidative stress and improving overall health.

Several studies have investigated the effects of dietary supplementation with citrus extracts or bioactive compounds on the antioxidant status of fish. For example, research by Mohammady *et al.* [18] demonstrated that feeding Nile tilapia with a diet containing fed essential oils extracted from sweet orange (*C. sinensis*) and lemon (*C. limon*) peels, significantly increased the activities of SOD, CAT, and GPx in the liver. Furthermore, the research of Harikrishnan *et al.* [29] investigated the effects of dietary supplementation with dried lemon peel on the antioxidant enzyme activities of grass carp. Results showed that lemon peel extract supplementation significantly upregulated the expression

of SOD, CAT and GPx levels, suggesting a potential enhancement of antioxidant defense mechanisms in Rohu (*Labeo rohita*).

CONCLUSIONS

In this study, it was observed that dietary supplementation with citrus species increased feed efficiency and growth performance by positively affecting the fish FCR. The beneficial effects of citrus-derived compounds on FCR were attributed to their ability to enhance nutrient digestibility, metabolic efficiency, hematologic and antioxidant status in fish. Harnessing the nutritional and bioactive properties of citrus fruits through dietary supplementation offers a potential strategy to improve feed utilization efficiency and mitigate production costs. According to the results of this study, the bioactive compounds present in citrus macerated oils, such as flavonoids and polyphenols, might enhance nutrient absorption and metabolic efficiency, leading to improved feed conversion in fish.

Current evidence suggests that citrus species have the potential to modulate the antioxidant system of fish through their bioactive compounds. Further research is needed to elucidate the mechanisms underlying these effects and to investigate the potential application of citrus-derived supplements in aquaculture practices aimed at improving fish health and welfare. It is especially important to adjust the appropriate doses of the macerated oils to be given according to the fish to be bred. Extracts, which are pharmaceutical precursor raw materials, are products that are not suitable for practical use, as they have a high probability of side effects and are costly as essential oils. Maceration products stand out as a desired feed additive product because they carry fat-soluble bioactive compounds, provided that the dose adjustment is made according to the fish species. In addition, being cost-effective is also a reason for preference for the manufacturer.

Conflicts of interest

The authors declare that I have no conflict of interest.

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