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Animal development, liver histology, and antioxidant activity in the muscle of zebrafish (*Danio rerio*) fed with natural additives in the diets

Desenvolvimento animal, histologia hepatica e atividade antioxidante do músculo de zebrafish (*Danio rerio*) alimentado com aditivos naturais na dieta

Desarrollo animal, histología hepatica y actividade antioxidante en músculo de zebrafish (Danio rerio) alimentado con aditivos naturales en la dieta

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Abstract

This work was carried out to evaluate the animal development, liver histology, and antioxidant effects on zebrafish fed with natural additives in different levels. Natural additives were composed by *Baccharis dracunculifolia* extract, cashew nut shell liquid (CNSL), and clove leaf essential oil (CLEO). The groups were: CONT: basal diet without the addition; CONB: addition of 50 g BHT kg^{-1} feed; MIX1: addition of 50 g of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: addition of 50 g of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: addition of 50 g of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: addition of 50 g of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). The average daily gain was lower for fishes fed the MIX4 diet. Diets effect was observed in liver histology ($P < 0.001$), which were greater in fishes fed CONB and MIX4 diets. Antioxidant activity in the diet evaluated using the ABTS assay was greater ($P < 0.05$) for treatments with natural additives. Similarly, antioxidant activity in zebrafish muscle ($P < 0.05$). Thus, the inclusion of 5% of natural additives in the diet composed by *B. dracunculifolia* extract, CNSL, and CLEO improved the antioxidant activity of zebrafish muscle.

Keywords: ABTS; Animal production; DPPH; Natural extracts; Phytochemicals.

Resumo

Este trabalho foi realizado para avaliar o crescimento animal, histologia hepática e efeitos antioxidantes em zebrafish alimentados com aditivos naturais em diferentes níveis. Os aditivos naturais foram compostos por extrato de *Baccharis dracunculifolia*, líquido da casca de castanha de caju (CNSL) e óleo essencial da folha do cravo da Índia (CLEO). Os grupos foram: CONT: dieta basal sem adição; CONB: adição de 50 g BHT/kg de ração; MIX1: adição de 50 g de aditivos naturais/kg de ração (60% *B. dracunculifolia*, 39% CNSL e 1% CLEO); MIX2: adição de 50 g de aditivos naturais/kg de ração (70% *B. dracunculifolia*, 29% CNSL e 1% CLEO); MIX3: adição de 50 g de aditivos naturais/kg de ração (90% *B. dracunculifolia*, 9% CNSL e 1% CLEO); e MIX4: adição de 50 g de aditivos naturais/kg de ração (99% *B. dracunculifolia*, 0,9% CNSL e 0,1% CLEO). O ganho médio diário foi menor para os peixes alimentados com a dieta MIX4. O efeito das dietas foi observado na histologia hepática ($P < 0,001$), que foi maior nos peixes alimentados com as dietas CONB e MIX4. A atividade antioxidante na dieta avaliada pelo ensaio ABTS foi maior ($P < 0,05$) para os tratamentos com aditivos naturais. Da mesma forma, a atividade antioxidante no músculo de zebrafish ($P < 0,05$). Assim, a inclusão de 5% de aditivos naturais na dieta composta por extrato de *B. dracunculifolia*, CNSL e CLEO melhorou a atividade antioxidante do músculo de zebrafish.
Palavras-chave: ABTS; DPPH; Extratos naturais; Fitoquímicos; Produção animal.

Resumen

Este trabajo se llevó a cabo para evaluar el crecimiento animal, la histología hepática y los efectos antioxidantes en zebrafish alimentados con aditivos naturales en diferentes niveles. Los aditivos naturales estaban compuestos por extracto de *Baccharis dracunculifolia*, líquido de cáscara de anacardo (CNSL) y aceite esencial de clavo (CLEO). Los grupos fueron: CONT: dieta basal sin adición; CONB: adición de 50 g BHT/kg de ración; MIX1: adición de 50 g de aditivos naturales/kg de ración (60% *B. dracunculifolia*, 39% CNSL y 1% CLEO); MIX2: adición de 50 g de aditivos naturales/kg de ración (70% *B. dracunculifolia*, 29% CNSL y 1% CLEO); MIX3: adición de 50 g de aditivos naturales/kg de ración (90% *B. dracunculifolia*, 9% CNSL y 1% CLEO); y MIX4: adición de 50 g de aditivos naturales/kg de ración (99% *B. dracunculifolia*, 0,9% CNSL y 0,1% CLEO). La ganancia diaria promedio fue menor para los peces alimentados con la dieta MIX4. El efecto de las dietas se observó en la histología del hígado ($P < 0,001$), que fue mayor en los peces alimentados con dietas CONB y MIX4. La actividad antioxidante en la dieta evaluada por el ensayo ABTS fue mayor ($P < 0,05$) para los tratamientos con aditivos naturales. Asimismo, actividad antioxidante en músculo de zebrafish ($P < 0,05$). Así, la inclusión de 5% de aditivos naturales en la dieta compuesta por extracto de *B. dracunculifolia*, CNSL y CLEO mejoró la actividad antioxidante del músculo de zebrafish.
Palabras clave: ABTS; DPPH; Extractos naturales; Fitoquímicos; Producción animal.

1. Introduction

Aquaculture is rapidly evolving. Genetic improvement oriented for growth over the years increased productive performance, but fish are more susceptible to diseases and to antioxidative stress. Antibiotics are widely used to prevent and treat diseases, and to increase animal performance (Miranda, Godoy, & Lee, 2018). However, there is growing concern on the emerging of antimicrobial resistance. Similarly, synthetic antioxidant additives, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ), frequently used in the food and livestock industry to improve antioxidant and antimicrobial activity (Raccach, 1984), are also a cause of concern (Maqsood, Benjakul, & Shahidi, 2013). Therefore, alternatives compounds are being developed.

Natural compounds can be originated from the secondary metabolism of plants and can be acquired from leaves, flowers, seeds, roots and barks (Sangwan et al., 2001). Natural compounds are usually obtained as crude extracts, vegetable and essential oils. Extracts and oils can be composed by few or numerous components in various concentrations, but usually the most abundant determines the biological properties and application of the extract or oil, such as bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, and antioxidative (Bakkali et al., 2008). Moreover, there is interest to explore and combine various sources of natural compounds to promote synergism and further improve biological properties (Chakraborty et al., 2014; Makkar et al., 2007). For example, combinations of caffeic and carnosic acids, or quercetin and rutin, demonstrated synergistic effects for antioxidant activity (Capitani et al., 2009).

Sources of natural compounds with potent biological properties are abundant and should be explored. Cashew (*Anacardium occidentale*) nut shell liquid (CNSL)is a byproduct composed by a mixture of anacardic acid, cardanol, traces of

cardol, and 2-methylcardol, with antibacterial activity (Trevisan et al., 2006). Clove (*Syzygium aromaticum*) leaf essential oil (CLEO) contains eugenol (4-alil-2-metoxifenol) as its main compound, which has strong insecticidal, antioxidant, antifungal, and antimicrobial activity (Mulla et al., 2017). *Baccharis dracunculifolia* is an endemic plant in South America enriched in terpenes, phenylpropanoids, specifically artepillin C (Bonin et al., 2019; Campos et al., 2016; Veiga et al., 2017).

Zebrafish (*Danio rerio*) has been proposed as a proxy for studies on immunology, toxicology (Sullivan & Kim, 2008), antioxidative status in food (Caro et al., 2016), nutrition and growth in fish (Ulloa et al., 2014), and physiology in mammals (Craig & Moon, 2011). Yet, natural additives having antimicrobial and antioxidant activity fed to zebrafish are still scarce. Thus, the aims of this study were to evaluate the animal development, liver histology, and antioxidant status of the muscle of zebrafish fed with different levels of natural additives (CNSL, CLEO and *B. dracunculifolia*).

2. Methodology

2.1 Diets and treatments

CNSL was purchased from Safeeds Animal Nutrition® (Cascavel, Brazil), and CLEO was purchased from Ferquima® (Vargem Grande Paulista, Brazil). *B. dracunculifolia* was collected in the region of Maringá (23°27'23.9"S 51°58'33.6"W), Paraná, south Brazil in August 2017. Herbarium specialists from Universidade Estadual de Maringá identified the species. Leaves, branches and stems (i.e., the plants without the roots) were dried in forced-air ovens at 40° C for 72 h and milled in Willey mill with through a 1-mm screen. Samples were stored in plastic containers at 4°C. A solution with the natural additives and ethanol (99.9% purity) was prepared (1:100; v:v) and filtered (filter paper (grammage – 80 g/m^2, thickness – 205 μm, pores – 14 μm).

A basal diet was obtained commercially and was formulated without synthetic antioxidants and natural additives (Table 1). BHT and natural additives were sprayed on the diet, according to treatments. Feed was stored at room temperature in dark containers during the experimental period.

The treatments were: CONT, basal diet without the addition of BHT and natural additives; CONB, basal diet with the addition of 50 g BHT/kg feed; MIX1, basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2 basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3, basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4, basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO).

Table 1. Formulation of the base diet used in the study.

Ingredients	Quantity (g kg^{-1})
Soybean meal	304.00
Ground corn	237.00
Corn gluten	145.00
Poultry flour	99.20
Feather flour	80.00
Rice flour	50.00
Dicalcium phosphate	49.50
Wheat gluten	10.00
L-Lysine	7.56
Common salt	5.00
Premix[a]	5.00
DL-Methionine	2.93
L-Threonine	1.30
Choline chloride	1.00
Antifungal	1.00
Vitamin C	1.00
L-Tryptophan	0.47

[a]Levels guaranteed per kilo of product: vit. A - 500,000 IU; vit. D3 - 200.00 IU; vit. E - 5,000 mg; vit. K3 – 1,000 mg; vit. B1 - 1,500 mg; vit. B2 - 1,500 mg; vit. B6 1,500 mg; vit. B12 - 4,000 mg; folic acid – 500 mg; calcium pantothenate – 4,000 mg; biotin – 50 mg; inositol - 10,000 mg; nicotinamide - 7,000 mg; choline - 40,000 mg; cobalt – 10 mg; copper – 500 mg; iron - 5,000 mg; iodine – 50 mg; manganese - 1,500 mg; selenium – 10 mg; zinc - 5,000 mg. Source: Authors.

2.2 Animals and facilities

All animal care and experimental procedures were conducted under the surveillance of the Animal Care and Use Committee of the Universidade Estadual de Maringá, Brazil (protocol no. 6119170518) and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

One hundred twenty zebrafish with 4 months of age (mean weight 362.65 ± 96.51 mg), reared by the PeixeGen Laboratory from the Universidade Estadual de Maringá were weighed using an analytical balance providing the initial body weight (IBW), and measured using a digital caliper providing the initial length (IL). Then, fishes were randomly distributed into six 24-L capacity aquariums (20 fishes per aquarium) with constant aeration, 14-hour light/10-hour dark cycles and temperature maintained between 26-28° C, as recommended for zebrafish. Partial aquarium water exchange (30%) was performed every three days. Aquarium water quality (Table 2) was verified every two days by measuring the pH, specific conductivity (µS cm^{-1}), total dissolved solids (mg L^{-1}), dissolved oxygen (mg L^{-1}), and temperature using a YSI Professional Plus Multi-Parameter Water Quality Meter.

Fish were subjected to a seven-day adaptation period before the beginning of the experimental period. Fish were fed at 09:00, 14:00, and 17:00 until apparent satiety was observed.

2.3 Animal development

Initial body weight and length was obtained in the day 1 of the trial. After 30 days, animals were euthanized by thermal shock in ice. Then, final body weight (FBW) and final length (FL) was obtained. FBW was used to estimate the average daily gain (ADG) using the following equation:

$$ADG = (FBW - IBW) / \text{days of the experiment}$$

2.4 Liver histology

Six fishes (three females and three males) of the treatments CONT, CONB, MIX3, and MIX4 were randomly selected for liver sampling. Animals were fixed in Bouin's solution for 2 hours, then livers were removed and placed in Bouin's

solution for 4 hours. Liver were stored in 70% ethanol. The material was then dehydrated through an ascending series of ethanol concentrations, diaphonized in xylol, and embedded in paraffin to obtain histological sections, which were stained with hematoxylin-eosin (Lewandowski et al., 2019). After staining, the slides were photographed under a 40X magnification Motic optical microscope (Motic BA310E) with Moticam 5.0MP camera. Hepatocyte count was performed using the Image Pro Plus software.

2.5 Dietary and muscle antioxidant activity

Fish were gutted and pooled to obtain three replicates by treatment with ~3 g per pool for the analysis of antioxidant activity in the muscle. Diet or muscle were combined with methanol (99.9% purity) to prepare a 1:1 solution (w:v) and homogenized in Ultra-turrax (IKA® - T10, USA) for 40 seconds. Then, samples were centrifuged at 4,000 rpm for 15 min, filtered, and used for analysis.

2.6 DPPH assay

The DPPH assay was performed according to Li et al., (2009), using 150 µL sample mixed with 2850 µL of a DPPH-containing methanolic solution (60 µM) and placed in a dark ambient environment for 30 min to react. Absorbance was measured at 515 nm using a spectrophotometer (Thermo Scientific™ Evolution 201 and 220 UV-Vis spectrophotometers, USA). Antioxidant activity was calculated as:

$$= (1 - (\frac{\text{Sample absorbance } t = 0}{\text{Sample absorbance } t}) \times 100$$

2.7 ABTS assay

The ABTS assay was performed according to Re et al. (1999) to obtain ABTS+ by the interaction of 7 mM ABTS (5 mL) with 140 mM potassium persulfate (88 µL). The mixture was incubated in the dark at room temperature for 16 h. The ABTS radical was diluted in ethanol to an absorbance of 0.70 ± 0.02. Samples (40 µL) were mixed with 1960 µL ABTS+ solution. After 6 min in the dark at ambient temperature, free radical sequestration activity was measured at 734 nm using a spectrophotometer (Thermo Scientific™ Evolution 201 and 220 UV-Vis spectrophotometers, USA). Antioxidant activity was calculated as:

$$= (1 - (\frac{\text{Sample absorbance } t = 0}{\text{Sample absorbance } t}) \times 100$$

2.8 Statistical analysis

The aquarium was considered the experimental unit for all analyses. Quantitative data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). All data were analyzed using the Satterthwaite approximation to determine the denominator df for tests of fixed effects. Model statements for initial and final BW and ADG contained the effects of treatment as an independent covariate. The covariance structure used was first order autoregressive, which provided the smallest Akaike information criterion and hence the best fit for all variables analyzed. All results are reported as covariate adjusted least square means, and the significance level was set at $P < 0.05$.

3. Results

Water quality was monitored throughout the study (Table 2). No difference ($P > 0.05$) was observed for pH, water specific conductivity and total dissolved solids among diets. However, tanks containing the MIX3 treatment had greater ($P <$

0.05) concentrations of dissolved oxygen (5.07 mg L^{-1}). Zebrafish can be affected when dissolved oxygen concentrations are lower than 4.0 mg L^{-1}, which was not the case for any treatment. There was no difference in temperature ($P > 0.05$) among treatments.

Table 2. Water quality parameters in aquariums with zebrafish (*Danio rerio*) fed natural additives.

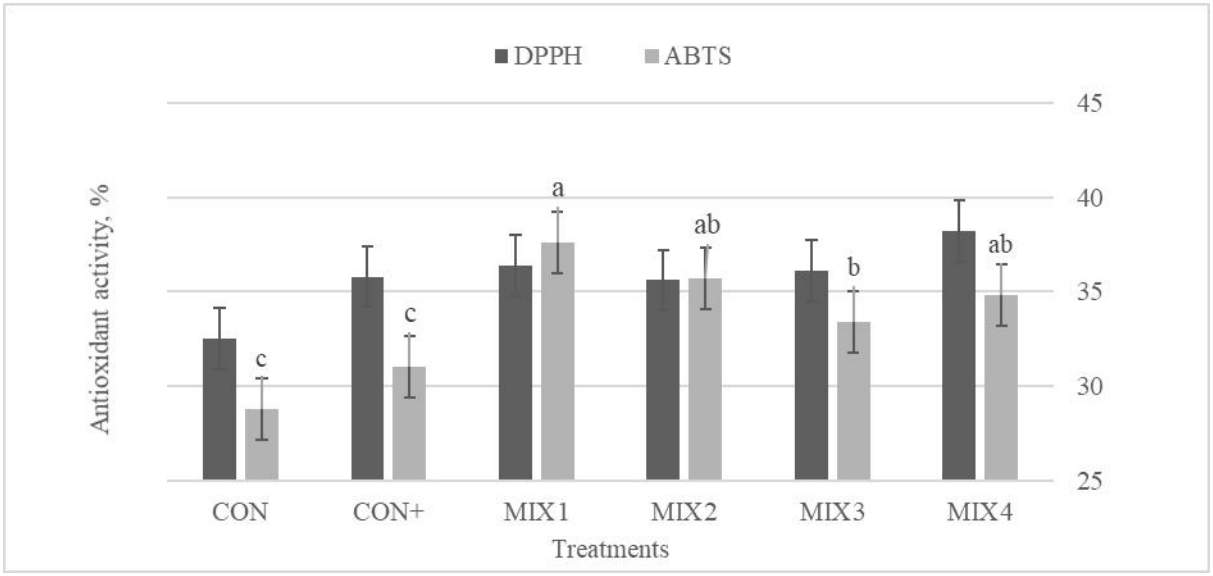
Parameters	CONT	CONB	MIX1	MIX2	MIX3	MIX4	SEM	*P*-value
pH	6.60	6.62	6.59	6.60	6.62	6.61	0.09	0.99
Specific conductivity, µS cm^{-1}	62.5	62.7	62.3	62.8	64.6	63.6	1.09	0.69
Total dissolved solids, mg L^{-1}	40.6	40.9	40.6	40.8	42.0	41.3	0.71	0.67
Dissolved oxygen, mg L^{-1}	4.39^{b}	4.31^{b}	4.51^{b}	4.74^{ab}	5.07^{a}	4.67^{ab}	0.09	0.01
Temperature, ºC	27.4	27.4	27.4	27.3	27.4	27.6	0.31	0.99

CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX1: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Means in the same line with different superscript are statistically different ($P < 0.05$). Source: Authors.

The antioxidant activity by the DPPH assay was similar ($P > 0.05$) for all the diets (Figure 1). On the other hand, CONT and CONB diets (without the inclusion of natural additives) had lower ($P < 0.05$) antioxidant activity compared to the diets with the inclusion of natural additives when the ABTS assay was performed (Figure 1).

The initial and final BW were similar ($P > 0.05$) when natural additives were included in the diet (Table 3). However, fishes fed MIX4 (blend composed by 99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO) had lower ADG ($P < 0.05$) when compared to fishes fed MIX3 diet (blend composed by 90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO) and MIX1 diet (blend composed by 60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO). Initial and final length were similar ($P < 0.05$) among fishes fed all diets.

Figure 1. Antioxidant activity of DPPH and ABTS free radicals in Zebrafish feed with natural compound addition.



CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX1: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Columns with letters: statistical difference between treatments. Source: Authors.

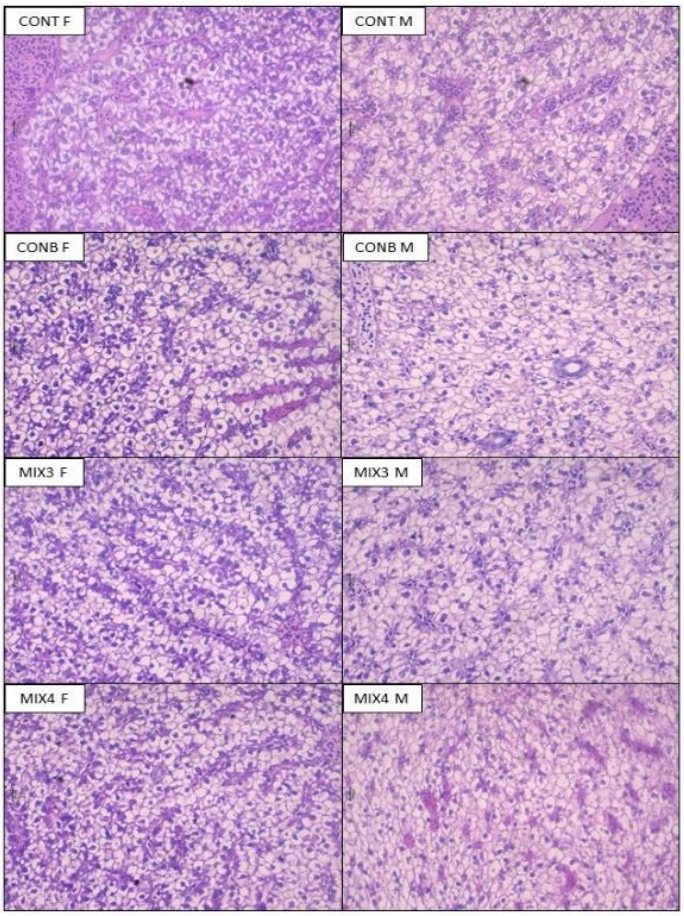
Table 3. Productive parameters of zebrafish (*Danio rerio*) fed with natural additives.

Parameters	CONT	CONB	MIX1	MIX2	MIX3	MIX4	SEM	*P*-value
Initial body weight, mg	361.00	383.68	340.50	365.00	358.00	353.68	46.45	0.85
Final body weight, mg	492.50	540.00	509.50	504.50	536.32	458.95	62.27	0.46
Average daily gain, mg day^{-1}	4.38^{ab}	5.21^{ab}	5.63^{a}	4.65^{ab}	5.94^{a}	3.51^{b}	0.82	0.01
Initial length, mm	32.4	33.1	32.1	32.5	32.5	30.3	0.64	0.07
Final length, mm	34.8	35.6	34.8	35.6	35.7	35.6	0.54	0.74

CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX1: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Means in the same column with different superscript are statistically different ($P < 0.05$). Source: Authors.

The hepatocytes quantity µm^{-1} was lower ($P < 0.001$) of female fed CONT and MIX3 (0.000122 and 0.000117) diets when compared to female fed CONB and MIX4 (0.000130 and 0.000128) diets, as showed in Figure 2 and Table 4. Males had similar results ($P < 0.001$), having both CONB and MIX4 diets (0.000120) greater quantities of hepatocytes compared to CONT and MIX3 (0.000110 and 0.000097) diets. MIX3 had lower quantities of hepatocytes when compared to CONT diet ($P < 0.001$).

Figure 2. Zebrafish histological slide photo fed diets with added natural compounds.



CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Source: Authors.

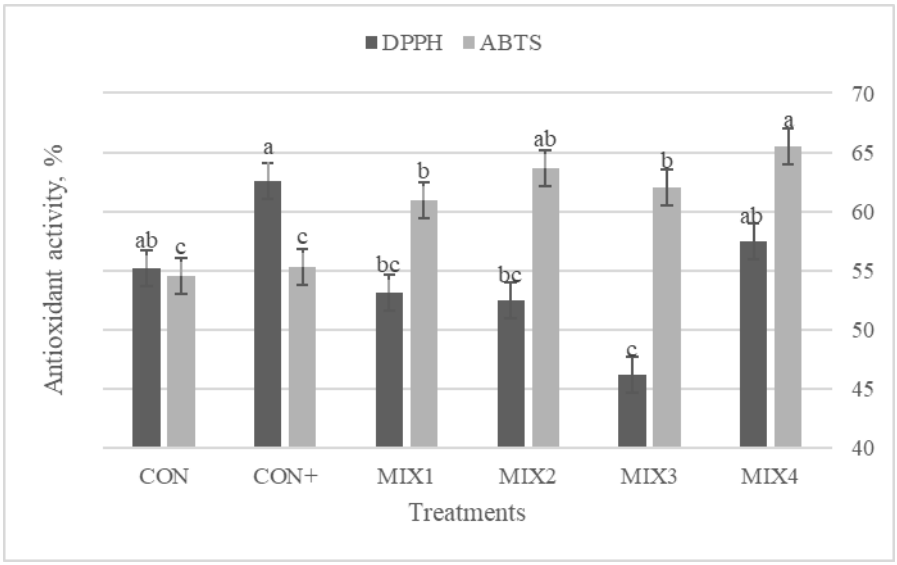
Table 4. Hepatocyte number per µm of zebrafish (*Danio rerio*) fed diets with natural additives.

Sexo	Treatments					
	CONT	CONB	MIX3	MIX4	SEM	*P*-value
Females	0.000122[b]	0.000130[a]	0.000117[b]	0.000128[a]	0.01	0.001
Males	0.000110[b]	0.000120[a]	0.000097[c]	0.000120[a]	0.01	0.001

CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX1: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Means in the same column with different superscript are statistically different ($P < 0.05$). Source: Authors.

The antioxidant activity in the muscle evaluated by the DPPH assay was affected ($P < 0.05$) by the diets (Figure 3). The fishes from CONB diet had highest antioxidant activity (62.6%), followed by fishes from MIX4 (57.4%), and CON (55.2%) diets. On the other hand, MIX2 and MIX4 had the highest ($P < 0.05$) antioxidant activity when evaluated by the ABTS assay, 63.6% and 65.5%, respectively.

Figure 3. Antioxidant activity of zebrafish muscle free radicals DPPH and ABTS fed diets with added natural compounds.



CONT: basal diet without the addition of BHT and natural additives; CONB: basal diet with the addition of 50 g BHT kg^{-1} feed; MIX1: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (60% *B. dracunculifolia*, 39% CNSL, and 1% CLEO); MIX2: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (70% *B. dracunculifolia*, 29% CNSL, and 1% CLEO); MIX3: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (90% *B. dracunculifolia*, 9% CNSL, and 1% CLEO); and MIX4: basal diet with the addition of 50 g blend of natural additives kg^{-1} feed (99% *B. dracunculifolia*, 0.9% CNSL, and 0.1% CLEO). Columns with letters: statistical difference between treatments. Source: Authors.

4. Discussion

The zebrafish has a fully sequenced genome, having 71% of its genes orthologous to those of humans (Vilella et al., 2008). Recently, there is great interest to use zebrafish as animal model in scientific research. Indeed, zebrafish has been used in studies for screening of novel natural products (Santos et al., 2016; Sullivan & Kim, 2008), evaluating antioxidant agents (Kirkwood et al., 2012) to determine biomarkers of oxidative stress-related processes (Caro et al., 2016), nutrition and growth of fishes (Ulloa et al., 2014). Data of growth and antioxidative status modulation can potentially be extrapolated to other species. Moreover, studies with zebrafish are usually short in duration and less resource consuming, which are highly desired characteristics in research.

There is evidence that natural additives can be improve performance of piglets (Muhl & Liebert, 2007), poultry (Khattak et al., 2014), fish (Naiel et al., 2020) and ruminants (Monteschio et al., 2017; Souza et al., 2019). Uncountable plants are yet to be evaluated and explored. For example, *B. dracunculifolia*, a plant harvested enriched in antimicrobial and used by bees to produce propolis (Rodrigues et al., 2020), has only recently being investigated. *B. dracunculifolia* extract decreased *in*

vitro growth of *Staphylococcus aureus*, *Bacillus subtilis* and *B. cereus* (Bonin et al., 2019). On the other hand, *B. megapotamica* var. *weirii* natural consumption by cattle (Driemeier et al., 2000; Stegelmeier et al., 2009) and 200 mg consumption of *B. pteronioides* by hamsters (Driemeier et al., 2000; Stegelmeier et al., 2009) have been reported as toxic. Despite no observed effect on FBW among treatments (Table 3), ADG was lower when the highest dose of *B. dracunculifolia* and the lowest doses of CNSL and CLEO were used (MIX4). The lower ADG is likely related to toxic effects of natural components of *B. dracunculifolia*, such as monoterpene phenols and sesquiterpene alcohols exhibit a strong cytotoxic (Fukuda et al., 2006).

The live is vital to detoxify and synthesize serum proteins such as albumin, fibrinogen, complement factors, and acute phase proteins (Menke et al., 2011). An increase in hepatocyte numbers per µm was observed when BHT and MIX4 were added to the diets (Table 4). Increased hepatocyte count may be related to increased liver metabolism to cope with toxic compounds (Zellmer et al., 2010), likely from the increased concentration of BTH in the CONB treatment, and potentially from the *B. dracunculifolia* concentration in the MIX4 diet (Jarvis et al., 1996; Varaschin & Alessi, 2003), which further supports the lower ADG observed in fishes fed MIX4 diet.

Two methods were tested to evaluate the antioxidant activity in feed and in the zebrafish muscle. DPPH and ABTS radicals have been widely used to evaluate the antioxidant properties of natural products because they are a source of free radicals that simulate reactive oxygen, nitrogen and peroxidized hydrogen species that affect biological systems (Bendary et al., 2013; Monteschio et al., 2017; Vital et al., 2016). The inclusion of natural additives in the diets was efficient to prevent antioxidant activity compared to no inclusion and were also more efficient compared to the inclusion of BTH, as observed in the ABTS assay. Thus, the incorporation of CNSL, CLEO, and *B. dracunculifolia* can be an alternative to the antioxidant activity of BTH in the diets. Similarly, the antioxidant activity in the muscle of zebrafish increased following the addition of the MIX compared to the CONT and CONB diets. Indeed, there is evidence of the transfer of antioxidant compounds from the diet to the muscle, improving animal heath, and extending shelf life of the product (Monteschio et al., 2017). As observed in this study, a higher percentage of free radical scavenging was observed for the fishes fed MIX4 diet, which supports that *B. dracunculifolia* promoted antioxidant activity. Antioxidants are typically added in feed at moderate levels; inclusion at high levels may lead to adverse effects such as pro-oxidative action (Martin, 2009).

5. Conclusion

The use of natural compounds such as B. dracunculifolia, cashew nut vegetable oil, and clove leaf essential oils can be used in the animal diet as a substitute for synthetic antioxidant compounds (BHT). Using zebrafish as an experimental model can improve animal growth parameters and protect animal diets and muscle from possible oxidation. However, attention should be paid to the concentrations of B. dracunculifolia used, due to its toxic effects.

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