




Flaxseed oil and clove leaf essential oil in Zebrafish diet (*Danio rerio*)

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ABSTRACT. Flaxseed oil is recognized as the plant source richest in α -linolenic acid, whereas clove leaf essential oil has a strong antioxidant capacity. The objective of this study was to determine the in vitro antioxidant capacity of diets containing a combination of flaxseed oil (FO) and clove leaf essential oil (CLEO), as well as to use zebrafish (*Danio rerio*) to assess their effect on the animals' growth. Fifty days after hatching, a total of 420 male specimens (0.29 ± 0.04 g) were divided into seven groups for each diet and fed for 55 days to be used, with the diets being: control, absent FO and CLEO; 3% FO + 0.5% CLEO; 3% FO + 1% CLEO; 6% FO + 0.5% CLEO; 6% FO + 1% CLEO; 9% FO + 0.5% CLEO and 9% FO + 1% CLEO. Antioxidant activity was determined through DPPH (2,2-diphenyl-1-picrylhydrazyl) tests, showing interaction effect between factors (FO x CLEO, $p < 0.05$); the diets containing 1% combined with 3, 6 or 9% of FO presented means higher than those of the 0.5% diets. No mortality was observed during the experiment. For final weight and weight gain, there was no interaction effect ($p > 0.05$), only isolated effect for FO, with the fish fed 6 and 9% diets having the best results. Final total length and specific growth rates showed interaction effect ($p < 0.05$). As for specific growth rates, the best response was that of the diet with 6% FO and 0.5% CLEO. Final length showed increase with FO levels, even when there was association with 0.5 or 1% of CLEO. Therefore, combined use of 9% of FL with 0.5% of CLEO is recommended for zebrafish.

Keywords: antioxidant; α -linolenic acid; DPPH; *Daniorerio*.

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Introduction

Fish, just as other vertebrates, cannot synthesize linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3), since they do not have the specific desaturase enzyme that turns them into essential fatty acids; therefore, both acids must be provided by means of diets to prevent nutritional deficiencies (Teitelbaum & Walker, 2011; Souza, Anido, & Tognon, 2007), for being fundamental to enable normal growth and animal survival (Sargent, Tocher, & Bell, 2002). Flaxseed oil differs from other vegetable oils for being the richest source of α -linolenic acid (Popa et al., 2012) and a precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are physiologically important with their immunological function (Nayak, Saha, Pradhan, Samanta, & Giri, 2017).

Excessive amounts of polyunsaturated fatty acids, such as α -linolenic, in diets can raise lipid unsaturation levels in fish tissues and make fish prone to attack by free radicals (EROS) (Kiron, Fukuda, Toshio, & Watanabe, 1995). These EROS, such as hydrogen peroxide, superoxide and hydroxyl, can attack the phospholipid membrane of the cells, react with cellular proteins and nucleic acids, and damage them, leading to immunosuppression (Sotoudeh, Kenari, Khodabandeh, & Khajeh, 2015).

To protect cells from damage, the organism has developed protective mechanisms, such as the action of antioxidant enzymes – catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and superoxide dismutase (SOD) (Halliwell & Gutteridge, 2007). When the EROS production rate is greater than the elimination capacity of the defense system, an exogenous antioxidant is required, which may come from the diet. In this regard, clove oil has the highest antioxidant capacity among commonly marketed essential oils (Teixeira et al., 2013).

The main constituent of clove oil is eugenol, to which many of the antioxidant properties are attributed (Ogata, Hoshi, Urano, & Endo, 200). Gülçin, Elmastaş, and Aboul-Enein (2012) showed that clove oil inhibited 97.3% of lipid peroxidation in linoleic acid emulsion at a concentration of 15 $\mu\text{g mL}^{-1}$. A protective effect of 0.5% clove oil in diets has been reported for carp fingerlings (*Labeo rohita*), through decreases in SOD activity and in lipid peroxidation levels (Asimi & Sahu 2016).

Zebrafish is an experimental model consolidated in several fields of research and, recently, has been considered as a model for aquaculture investigation in nutrition, reproduction and wellbeing studies (Ulloa, Medrano, & Feijoo, 2014; Ribas & Piferrer 2014). The advantages of this model include ease of management as to breeding, high reproductive capacity, rapid development and sequenced genome (Lawrence, 2017).

The objective of this study was to determine the in vitro antioxidant capacity of diets containing a combination of flaxseed oil (FO) and clove leaf essential oil (CLEO) through DPPH (2,2-diphenyl-1-picrylhydrazyl) tests, then use zebrafish (*Danio rerio*) to assess their effect on the growth of the animals fed with these diets.

Material and methods

The experiment was run at the Ornamental Fish Laboratory of PeixeGEN Research Group – Management, Improvement and Molecular Genetics in Freshwater Pisciculture of the State University of Maringá [Universidade Estadual de Maringá] - UEM, for a period of 55 days.

This project was approved by the Ethics Committee on Use of Animals [Comitê de Ética no Uso de Animais] (CEUA) of the State University of Maringá, under protocol No 8851180216 of May 2016.

Experimental diets and diet management

Seven experimental diets were prepared according to the nutritional recommendations proposed by Siccardi et al. (2009), with the following inclusion levels of flaxseed oil (FO) and clove leaf essential oil (CLEO): Diet 1 (Control, without inclusion of FO and CLEO); Diet 2 (3% of FO + 0.5% CLEO); Diet 3 (3% of FO + 1% CLEO); Diet 4 (6% of FO + 0.5% CLEO); Diet 5 (6% of FO + 1% CLEO); Diet 6 (9% of FO + 0.5% CLEO); Diet 7 (9% of FO + 1% CLEO). Diet composition and fatty acid profile (determined according to Figueiredo et al. (2016)) are shown in Tables 1 and 2.

Corn oil was used for keeping the diets isoenergetic. The ingredients were ground using a hammermill with a sieve measuring 0.3 mm in diameter. The feed was processed through an Ex-Micro® extruder measuring 1.0 mm in diameter. The fish were fed four times a day (8, 11, 14 and 17h) until apparent satiety.

Table 1. Percentage and centesimal composition of the experimental diets.

Ingredients	Inclusion levels (%)						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Soy protein isolate	50.90	50.90	50.90	50.90	50.90	50.90	50.90
Corn	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Corn gluten meal	7.06	7.06	7.06	7.06	7.06	7.06	7.06
Corn oil	10.00	6.50	6.00	3.50	3.00	0.50	0.00
FO	0.00	3.00	3.00	6.00	6.00	9.00	9.00
CLEO	0.00	0.50	1.00	0.50	1.00	0.50	1.00
Broken rice	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate	3.63	3.63	3.63	3.63	3.63	3.63	3.63
Lysine	1.14	1.14	1.14	1.14	1.14	1.14	1.14
¹ Mineral and vitamin supplement	1.00	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Calcitic Limestone	0.41	0.41	0.41	0.41	0.41	0.41	0.41
Common salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Tryptophan	0.09	0.09	0.09	0.09	0.09	0.09	0.09
<i>Centesimal composition</i>							
Crude protein	51.82	51.26	51.15	50.97	51.06	50.85	51.47
Energy (kJ g ⁻¹)	17.16	17.28	17.57	17.23	17.41	17.68	17.13
Ethereal extract	11.26	11.09	11.52	11.63	11.06	11.81	11.32
Calcium	1.10	1.26	1.03	1.16	1.15	1.32	1.01
Total phosphorus	1.33	1.14	1.29	1.22	1.17	1.55	1.10

¹Guaranteed levels per kilo of product: vit. A - 500,000 IU; vit. D3 - 200,000 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 - 4000 mg; biotin - 50 mg; inositol - 10,000; nicotinamide - 7,000; 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.

Table 2. Fatty acid profile of the experimental diets (% of total fatty acids).

Fatty acids	Experimental diets						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
16:00	11.56	10.81	11.01	9.29	9.80	7.95	8.57
18:00	4.87	5.51	5.14	5.08	5.46	5.40	5.45
20:00	0.54	0.54	0.46	0.42	0.43	0.44	0.38
22:00	0.63	0.59	0.51	0.44	0.45	0.43	0.31
24:00	0.26	0.26	0.28	0.22	0.22	0.23	0.24
18:1n-9	28.31	31.11	26.40	29.78	31.06	31.36	27.95
18:1n-7	1.49	1.42	1.05	1.15	1.13	1.12	0.69
20:1n-9	0.33	0.34	0.24	0.27	0.29	0.28	0.20
18:2n-6	46.58	37.69	36.67	30.17	29.44	27.80	19.44
18:3n-3	5.43	11.71	12.89	23.17	21.69	24.99	27.65
¹ ΣSat	17.85	17.72	17.40	15.46	16.37	14.45	14.95
² ΣMon	30.13	32.87	27.69	31.21	32.48	32.76	28.83
³ ΣPol	52.01	49.41	49.55	53.34	51.13	52.79	47.09
n6/n3	8.57	3.22	2.85	1.30	1.36	1.11	0.70

¹Sum of saturated fatty acids; ²Sum of monosaturated fatty acids, and ³Sum of polyunsaturated acids.

Fish and experimental conditions

A total of 420 male zebrafish (*D. rerio*) aged 50 days after hatching were used; they weighed 0.29 ± 0.04 g on average and had an average total length of 30.67 ± 0.71 mm. The animals were distributed into glass tanks with capacity for 50 liters, individually equipped with an internal filter, a 50w thermostat and constant aeration by means of a central air blower.

The average water temperature, pH and dissolved oxygen were $26.23 \pm 0.54^\circ\text{C}$, 7.3 ± 0.01 and 6.6 ± 0.32 mg L⁻¹, within the comfortable range for the species (Westerfield, 2007). At the end of the experiment, the fish were euthanized (MS-222 tricaine methanesulfonate in 250 mg L⁻¹ for 10 minutes) to have their weight (g) and length (mm) taken.

Growth of the animals

The growth calculations performed for the animals were:

- Survival rate: Fn (final number of fish); In (initial number of fish).

$$\text{SR}\% = \frac{\text{Fn} * 100}{\text{In}}$$

- Weight gain: Fw (final weight); Iw (initial weight).

$$\text{WG} = \text{Fw} - \text{Iw}$$

- Specific growth rate: lnFw (final weight natural log); lnIw (initial weight natural log); Nd (number of experiment days).

$$\text{SGR} = \left[\left(\frac{\ln\text{Fw} - \ln\text{Iw}}{\text{Nd}} \right) * 100 \right].$$

In vitro determination of antioxidant activity in the experimental diets

The antioxidant activity of the diets was determined through DPPH (2,2-diphenyl-1-picrylhydrazil) tests, following the Quencher procedure (Hissin & Hilf, 1976).

Statistical analysis

Data were subjected to factorial analysis of variance. In case of significant differences, Tukey's test was run in a factorial split design at 5% probability level. Dunnett's test (5% probability level) was used for comparison with the control group. Calculations were performed on Statistical Analysis System [SAS] version 9.3 (2011).

Results

Determining the antioxidant capacity of the diets

Results show interaction effect between factors ($p < 0.05$); diets containing 1% of CLEO, even when combined with 3, 6 or 9% of FO, presented means higher than those of diets with 0.5%. In addition, the

association of FO and CLEO caused a significant increase ($p < 0.05$) in antioxidant capacity compared to the control diet, with an average increment of 270% (Table 3).

Table 3. Determination of antioxidant capacity in diets containing flaxseed oil and clove leaf essential oil, both analyzed through DPPH (2,2-diphenyl-1-picrylhydrazil) tests.

Response	p Value	Flaxseed oil (%)	Clove leaf essential oil (%)		
			0.00	0.50	1.00
DPPH	<0.001*	0.00	13.70±0.13	-	-
		3.00	-	37.28±1.27 Bb**	49.91±1.51 Aa**
		6.00	-	41.52±1.44 Ab**	49.93±1.75 Aa**
		9.00	-	37.84±1.75 Bb**	51.43±0.97 Aa**

Data are presented as mean ± standard deviation. Values followed by distinct uppercase letters in the column and distinct lowercase letters in the row are statistically different ($p < 0.05$). (*) indicates interaction effect ($p < 0.05$). (**) differs significantly from control by Dunnett's test ($p < 0.05$).

Growth of the animals

No mortality was observed during the experiment, so survival stood at 100% in all treatments. For final weight and weight gain, there was no interaction effect between factors ($p > 0.05$), only isolated effect ($p < 0.05$) for FO; the animals fed diets 6 and 9% had the best results (Table 4). Fish fed with FO and CLEO showed improvement compared to the control group.

Table 4. Evaluation of growth characteristics in zebrafish (*Danio rerio*) fed with experimental diets containing flaxseed oil (FO) and clove leaf essential oil (CLEO), showing isolated effect between factors.

Components	Response	
	WG (g)	FW (g)
Effects		
Flaxseed	0.017*	<.0001*
Clove	0.122ns	0.122ns
Factors		
Control	0.08±0.01	0.37 ± 0.01
FO		
3%	0.15±0.03 b**	0.44±0.03 b**
6%	0.22±0.05 a**	0.50±0.06 a**
9%	0.21±0.03a**	0.50±0.04 a**
CLEO		
0.5%	0.21±0.06**	0.50±0.06**
1%	0.17±0.01**	0.46±0.03**

Data are presented as mean ± standard deviation. Values followed by distinct letters in the column are statistically different ($p < 0.05$). (*) indicates isolated effect of the factors ($p < 0.05$). (**) differs significantly from control by Dunnett's test ($p < 0.05$).

Final total length (FL) and specific growth rate (SGR) showed interaction effect ($p < 0.05$) (Table 5). For SGR, the best response was found with the diet containing 6% of FO and 0.5% of CLEO. FL increased with FO levels, even when there was association with 0.5 or 1% of CLEO.

Table 5. Growth characteristics in zebrafish (*Danio rerio*) fed with experimental diets containing flaxseed oil and clove leaf essential oil, showing interaction effect between factors.

Response	p Value	Flaxseed oil (%)	Clove leaf essential oil (%)		
			0.00	0.50	1.00
SGR (% of weight day ⁻¹)	0.002*	0.00	0.46±0.07	-	-
		3.00	-	0.74±0.15 Ba	0.78±0.14 Aa
		6.00	-	1.15±0.15 Aa**	0.85±0.09 Ab**
		9.00	-	1.04±0.13 ABa**	0.93±0.12 Aa**
FL (mm)	<0.001*	0.00	31.07±1.65	-	-
		3.00	-	33.23±1.29 Bb	34.16±1.59 Bb**
		6.00	-	34.79±1.12 ABa**	34.23±1.29 Ba**
		9.00	-	36.38±1.35 Aa**	36.89±1.02 Aa**

Data are presented as mean ± standard deviation. Values followed by distinct uppercase letters in the column and distinct lowercase letters in the row are statistically different ($p < 0.05$). (*) indicates interaction effect ($p < 0.05$). (**) differs significantly from control by Dunnett's test ($p < 0.05$).

Discussion

The increased antioxidant capacity of the diets is related to the chemical composition and antioxidant property of CLEO, which is mainly made up of eugenol (81 to 86%), its main bioactive representative (Sohilait, 2015). Moreover, Jirovetz et al. (2006) found β -caryophyllene (17.4%) and α -humulene (2.1%) in this oil, which have antioxidant activity. This action was tested by Gaspar, Duarte & Santana (2018) in diseases associated with inflammation and oxidative stress, which led to a decrease in reactive oxygen species due to its effect of eliminating free radicals against hydroxyl anions, superoxide anions, and lipid peroxidation.

The research conducted by Gülçin et al. (2012) confirms its antioxidant activity by reducing DPPH radicals to concentrations lower than those of hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). In addition, the high antioxidant capacity of CLEO was shown by Biondo et al. (2017), when they analyzed 18 samples of essential oils, with results ranking CLEO first (1753.36 $\mu\text{mol TE/g}$) and cinnamon essential oil second (779.06 $\mu\text{mol TE g}^{-1}$).

The fish adapted well to the diets and experimental conditions, as there was no mortality during the study period with 55 days of feeding. These results are similar to those reported by Araújo et al. (2017), who also did not detect mortality in zebrafish subjected to a 5-month feeding test assessing flaxseed, olive, fish and corn oils.

With respect to growth, the result of the present study corroborates with reports in the literature that lipid increase at an adequate level can improve fish growth, since requirements as to energy and, mainly, essential fatty acids, such as α -linolenic acid, are met (Ikeda et al., 2011). Flaxseed oil with a high dietary content increased α -linolenic acid (n-3) in the diet, positively impacting the growth of the zebrafish, possibly showing the animal's high capacity to digest and absorb this component. The study carried out by Meinelt, Schulz, Wirth, Kürzinger, and Steinberg (2000) with zebrafish also found positive growth when the latter were fed diets containing 14 to 47% of n-3 fatty acids.

Clove leaf essential oil in the diet is also believed to have contributed to the greater retention of α -linolenic acid present in flaxseed oil, performing its antioxidant function by inhibiting or delaying lipid peroxidation, protecting it against the harmful action of free radicals, thus improving its assimilation and leading to the positive growth of the zebrafish. Sotoudeh et al. (2015) reported a similar result for trout (*Salmo trutta caspius*), according to which α -tocopherol protected EPA and DHA against oxidation in the cell membrane, allowing for a greater incorporation of these acids and improving the development of the fish. In this sense, ingestion of antioxidants such as clove leaf essential oil is fundamental in diets with high contents of polyunsaturated fatty acids in order to stabilize the lipid peroxidation process.

Conclusion

The antioxidant capacity of the diets increased with the addition of clove leaf essential oil. The growth of the zebrafish improved with the addition of flaxseed oil and clove leaf essential oil; thus, a combined use of 9% and 05% of said oils, respectively, is recommended.

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