

Use of the essential oil of *Thymus vulgaris* (thyme) and its nanoemulsion as an anesthetic during the cultivation of tambaqui (*Colossoma macropomum*): Anesthesia induction and recovery curve, physiology, performance and feed consumption

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Abstract

The thymol chemotype of the essential oil of *Thymus vulgaris* (EOTV-T) and its nanoemulsion (NEOTV-T) were investigated as anesthetics in the management of *Colossoma macropomum*. The stability of nanoemulsions was analyzed by zeta potential and dynamic light scattering for an interval of 37 days prior to carrying out two anesthesia experiments. The first experiment determined anesthesia induction and recovery intervals for juveniles anesthetized with different concentrations of EOTV-T and NEOTV-T. Based on the obtained induction and recovery curve, the second experiment adopted concentrations of 75 mg/L of EOTV-T and 50 mg/L of NEOTV-T for use during biometric management every 10 days for 60 days of cultivation of juvenile *C. macropomum*. The results showed that NEOTV-T had an average diameter of less than 100 nm for up to 22 days and was more effective at inducing anesthesia than EOTV-T. The continuous use of anesthetics during biometrics did not affect juvenile performance in terms of growth, feed consumption and survival. Furthermore, no significant differences were observed in hematological and biochemical parameters between juveniles of the control treatment and those subjected to biometric management using 75 mg/L of EOTV-T and 50 mg/L of NEOTV-T. It can be concluded that the essential oil of *T. vulgaris* and its nanoemulsion can be used to facilitate the management of juvenile *C. macropomum* without compromising their performance or well-being. More studies need to be carried out to improve the stability of nanoemulsions of the essential oil of *T. vulgaris* and to evaluate their effects on acute stress.

1. Introduction

Tambaqui, *Colossoma macropomum* (family Characidae), is a species of freshwater fish originating from the Amazon basin that is of economic importance in aquaculture (Brian et al. 2004). This scaled fish has dark spots on the ventral region (Boaventura et al. 2021), a robust body and tasty meat and can reach considerable sizes (Wojnárovich and Van Anrooy. 2019). The majority of *C. macropomum* production is in the northern region of South America, and it is the second most produced fish species in Brazil (Peixe BR. 2023). Due to its performance in captivity, its production has expanded to several tropical Asian countries, including China, Indonesia, Malaysia, Myanmar and Vietnam (Wojnárovich and Van Anrooy. 2019).

Concern for animal welfare and the search for sustainable aquaculture practices has led to increased exploration of the use of essential oils to replace synthetic anesthetics during fish handling procedures (Aydın and Barbas. 2020; Purbosari et al. 2019). The use of these substances helps prevent physical damage during handling and even mitigates fish stress responses (Ross et al. 2009; Stoskopf and Posner. 2008). *Thymus vulgaris*, popularly known as thyme, is a plant species of the family Lamiaceae (Azad et al. 2014). The essential oil extracted from this plant can present different types of major components, such as thymol, which is a promising substance for use as an anesthetic for several species of fish (Azad et al. 2014; Bagamboula et al. 2004; Brandão et al. 2021; Yousefi et al. 2018), even tambaqui (Boaventura et al. 2022a).

In addition to the use of traditional essential oils, several studies have evaluated the use of their nanoparticles or nanoemulsions as anesthetic agents (Ferreira et al. 2020; López-Cánovas et al. 2020; Rodrigues et al. 2021; Yostawonkul et al. 2019). The use of nanoparticles and nanoemulsions obtained from these essential oils increases their solubility in water, which improves their absorption and thus reduces the amount of substance needed to induce anesthesia, thereby mitigating possible side effects in fish (Ferreira et al. 2020; López-Cánovas et al. 2020). Despite these studies using essential oils and their nanoemulsions as anesthetics, no study has evaluated the influence of the constant use of these substances during biometric management and their possible effects on animal performance.

Therefore, this study aimed to determine the ideal concentrations of the essential oil of *T. vulgaris* and its nanoemulsion to be used during biometric management of juvenile *C. macropomum* by evaluating the effects of their continuous use during cultivation on feed consumption, performance, physiology and metabolism.

2. Material and methods

2.1 Thymol chemotype of the essential oil of Thymus vulgaris (EOTV-T)

The thymol chemotype of the essential oil of *Thymus vulgaris* used in the experiments was acquired from the company Laszlo (<https://www.emporiolaszlo.com.br>), which also provided its composition characterized by gas chromatography (Table 1).

Table 1
Composition of the essential oil of *Thymus vulgaris*,

Composition	(%)
α - thujeno	0.6
α -pineno	1.4
canphene	0.9
β -pineno	0.1
myrcene	1.6
p-cymene	31.2
limonene	0.2
1.8 cineole	1.2
γ -terpinene	5.4
linalool	0.7
tymol	55.9
β -caryophyllene	0.3
The essential oil of <i>Thymus vulgaris</i> was acquired from the company Laszlo Aromoterapia LTDA, which also supplied their composition, obtained using a HP 5890 gas chromatograph. Peaks less than 0.1% were disregarded.	

2.2 Preparation of the nanoemulsion of the thymol chemotype of the essential oil of *Thymus vulgaris* (NEOTV-T).

The nanoemulsion of the essential oil of *Thymus vulgaris* (NEOTV-T) was prepared following the method described by Candido et al. (2022), with adaptations. Initially, an emulsion was prepared containing 5%w/w of EOTV, 1%w/w of Tween 80 and 94%w/w of deionized water. The water and oils were first mixed using a mechanical mixer (Ultraturrax, IKA T10) at 14,500 rpm for 10 minutes at a temperature of 27°C. For nanoemulsion, the emulsion was then subjected to an ultrasonic tip sonicator (Ultronique, Desruptor, Indaiatuba-SP) operating at 70% amplitude, using a 4 mm diameter probe immersed halfway into the emulsion. Five cycles of 4 minutes (2 minutes on and 2 minutes off) were used during the process to avoid heating the emulsion.

2.3 Characterization of the nanoemulsion of the thymol chemotype of the essential oil of *Thymus vulgaris*.

2.3.1 Dynamic Light Scattering (DLS) and Zeta Potential

Diameter measurements were made with DLS and zeta potential of *T. vulgaris* nanoparticle samples at a concentration of 0.4 mg/mL. Both DLS and zeta potential tests were performed with ZetaPALS equipment (Brookhaven Instruments). Analyses were performed on days 1, 2, 3, 6, 15, 22 and 37 after sample preparation. Means and standard deviations were obtained by taking 30 readings of DLS and Zeta Potential for each analysis day.

2.4. Animals and laboratory acclimatization

Three hundred and ten juvenile *C. macropomum* ($2.78 \pm 0.88\text{g}$ and $5.43 \pm 0.61\text{ cm}$) were distributed and acclimatized in 30 L tanks filled with 28 L of water and maintained in recirculating aquaculture systems (RAS) for 7 days. During acclimatization, the juveniles were fed an extruded commercial diet (40% crude protein, WeaN Prime 1.8 to 2.3 mm – Bernaqua) twice a day (08:00 and 16:00 h) until apparent satiety. Water quality parameters were measured daily during this period, and were as follows: temperature ($28.9 \pm 0.2^\circ\text{C}$) and pH (7.05 ± 0.21) were measured using a COMBO portable pH meter (HANNA-HI 98107), dissolved oxygen ($6.33 \pm 0.27\text{ mg/L}$) by a digital oximeter (HANNA-HI9146), and total ammonia ($0.25 \pm 0.00\text{ mg/L}$) using Alcon colorimetric kits.

2.5. Experimental design

Two experiments were carried out at the Laboratório de Aquicultura of the Universidade Federal de Minas Gerais (UFMG). The methodology was approved by the Comissão de Ética no Uso de Animais of UFMG, process CEUA: 37/2024.

The anesthetic solutions were prepared by weighing the essential oil of *T. vulgaris* (EOTV-T) and diluting it 1:10 (w/w) in 99% ethanol. The nanoemulsion (NEOTV-T) was diluted directly in water.

2.5.1 Experiment 1. Anesthesia induction and recovery for *Colossoma macropomum* undergoing anesthesia with EOTV-T and NEOTV-T

One-hundred and thirty juvenile *C. macropomum* were fasted for 24 hours. Anesthesia induction time was evaluated using 13 2-L aquariums, one being the control (containing water with the same amount of alcohol used to dilute the highest concentration of EOTV-T tested), six with different concentrations of EOTV-T (50, 75, 100, 150, 200 and 300 mg/L) and another six with different concentrations of NEOTV-T (50, 75, 100, 150, 200 and 300 mg/L). Another 13 2-L aquariums containing only clean water were used to determine anesthesia recovery time.

Ten juveniles were used for each concentration tested. Induction and recovery times were determined according to the criteria described by (Schoettger and Julin. 1967), whereby induction time was determined when a juvenile presented loss of balance and absence of external stimuli, while recovery time was determined through recovery of these responses. After recovery, the juveniles were transferred again to their respective RAS tanks for determination of survival rate 24 hours after the procedure.

2.5.2 Experiment 2. Performance and physiological responses of juvenile *Colossoma macropomum* subjected to constant biometric management with and without the use of EOTV-T and NEOTV-T

Based on the results of Experiment 1, a new experiment was carried out with three treatments for biometrics with four repetitions each: control (0 mg/L), 75 mg/L of thyme essential oil (EOTV-T) and 50 mg/L of thyme nanoemulsion (NEOTV -T). These concentrations were chosen because they present induction and recovery intervals that do not differ significantly from those indicated in the literature (less than 3 minutes for induction and less than 5 minutes for recovery) (Keene et al. 1998; Marking and Meyer. 1985). One-hundred and eighty juveniles ($2.78 \pm 0.88\text{g}$ and $5.43 \pm 0.61\text{ cm}$) were used for this experiment. Before starting the experiment, the juveniles underwent biometric procedures with and without the use of anesthetics. The juveniles were then distributed in 12 150-L tanks (useful volume of 100 L) at 15 fish/tank kept in a RAS where they were cultivated for 60 days. Biometrics were performed every 10 days. The NEOTV-T used in the biometric procedures was used within 20 days of preparation.

During the experiment, juveniles were fed until apparent satiety twice a day (08:00 and 16:00 h) with the same extruded commercial diet used during acclimatization. The food provided was previously weighed for each tank. Leftovers were collected 30 minutes after feeding, which were dried and weighed to estimate consumption and feed conversion.

After 60 days, all juveniles were weighed and measured (without the use of anesthesia) for blood collection.

Water quality parameters relating to temperature and pH were measured daily using a multi-parameter probe (HANNA brand, model HI98129). Dissolved oxygen (DO) and ammonia were measured every 3 days using a digital oximeter (HANNA brand, model HI9146-04) and colorimetric kits from Alcon, respectively.

Biometric procedures were performed individually for each repetition every 10 days and at the end of 60 days. During biometrics, the juveniles were captured in the culture tanks with the aid of a pulsar and placed in a 20-L bucket (filled with 10 L of water from the culture tank itself) containing or not the addition of anesthetic (EOTV-T or NEOTV-T). The juveniles were collected individually in the bucket, contained in a damp cloth, weighed using a semi-analytical scale (Marte AD 2000 0.01 g) and measured for total length with a ruler to determine final weight (PF) and total length (TC). After the biometrics process, the juveniles were returned to the cultivation tank where recovery from anesthesia took place. Biometric data and feed consumption were used to calculate the following:

- Weight gain (WG) $((\text{final weight} - \text{initial weight})/\text{initial weight}) \times 100$;
- Feed consumption (FE.C (g) = (offered food (g) - dry leftover food (g))/ number of animals);
- Apparent feed conversion (FCR) (feed consumption/weight gain).

Survival was also determined every 10 days and at the end of the experiment. The survival rate was determined by counting the number of initial and final animals in each phase and calculating the

following:

Survival (%) = (number of live fish x 100) / (total initial number of fish per tank)

Fish physiology and stress were evaluated by collecting blood from 12 animals of each treatment, 3 from each repetition. Two collections were made from each animal: the first collected about 1 mL (0.7 mL using heparinized syringes, to which 10% heparin was subsequently added; the second collected about 0.3 mL in syringes without heparin). Part of the aliquots containing heparin were used to determine hemoglobin (Hb) (Quibasa-Bioclin, Belo Horizonte, MG, Brazil) and hematocrit (HTC) using the micro-hematocrit method with capillary tubes centrifuged in a microcentrifuge followed by reading in an appropriate table (Goldenfarb et al. 1971).

Serum (without heparin) was used to determine total plasma protein (TPP) and albumin. The remainder of the collected blood was centrifuged at 4000 rpm for 5 minutes, after which the plasma was separated and used for biochemical analyses and determination of glucose (GLU), triglycerides (TRY), cholesterol (CHO) and the enzymes alanine aminotransferase enzymes (ALT) and aspartate aminotransferase (AST), using commercial colorimetric kits (Quibasa-Bioclin, Belo Horizonte, MG, Brazil) followed by reading on a spectrophotometer (Bioclin 100® semi-automatic analyzer).

2.6. Statistical analysis

All data were subjected to the Shapiro-Wilk normality test and Levene's test for homoscedasticity. Anesthesia induction and recovery times were subjected to regression analysis. Parametric data were analyzed by One-way ANOVA followed by Tukey's test at 5% probability. Data regarding Zeta Potential, pH, hematocrit and albumin were classified as non-parametric and were analyzed using the Kruskal-Wallis test followed by Dunn's post-test at 5% probability. SigmaPlot and Infostat software were used for data analysis.

3. Results

3.1. Dynamic Light Scattering (DLS) and Zeta Potential

The average diameter of the particles present in the nanoemulsion increased during storage, but did not exceed 15nm after 35 days of storage. (Fig. 1A). The zeta potential of the particles was approximately -19mV and reduced slightly to -10mV after 35 days of storage. A decrease in zeta potential contributes to increasing the average diameter of nanoparticles due to instability processes such as coalescence observed in nanoemulsions with zeta potential lower than 35mV in modulus. The zeta potential of the particles remained unchanged in the last days of storage, day 23 to day 37 (Fig. 1B) ($P > 0.05$).

Experiment 1.

3.2. Anesthesia induction and recovery times

No mortality was observed during nor 24 hours after anesthesia. The induction (Fig. 2A and 2C) and recovery (Fig. 2B and 2D) curves for the juveniles and the average data analyzed by ANOVA (Table 2) show that the increase in EOTV-T concentration reduced induction time until the concentration of 150 mg/L.

Table 2

Induction and recovery times for juvenile tambaqui (*Colossoma macropomum*) subjected to different concentrations of the essential oil of *Thymus vulgaris* and its nanoemulsion.

Concentration mg/L	Induction time (s)		Recuperation time (s)	
	EOTV	NEOTV	EOTV	NEOTV
50	201.4 ± 23.44aA	116.11 ± 5.35bA	94.85 ± 11.53aC	109.86 ± 18.11aD
75	95.89 ± 12.44aB	76.5 ± 7.20bB	131.87 ± 26.40aCB	118.50 ± 19.98aD
100	51.5 ± 6.15aC	47.49 ± 6.30aC	135.25 ± 35.05aCB	123.28 ± 11.65aD
150	34.9 ± 6.20aD	34.00 ± 6.68aD	113.62 ± 17.45bC	170.14 ± 32.52aC
200	23.90 ± 5.08aD	26.44 ± 3.61aE	167.84 ± 31.00bB	260.83 ± 41.52aB
300	24.55 ± 3.35aD	23.9 ± 1.20aE	286.00 ± 32.68bA	382.16 ± 31.25aA
Results expressed as Means ± Standard Deviation. Thymol chemotype of the essential oil of <i>Thymus vulgaris</i> (EOTV-T) and its nanoemulsion (NEOTV-T). Results followed by different lowercase letters indicate a significant difference between treatments at the same concentration. Results with different capital letters indicate a significant difference between concentrations of the same treatment by ANOVA followed by Tukey's test (P < 0.05).				

Juveniles anesthetized with 50, 75, 100 and 150 mg/L of EOTV-T had similar anesthesia recovery times, while those anesthetized with 200 mg/L of EOTV-T had a longer recovery time than did those anesthetized with 50 mg/L. The longest recovery time using EOTV-T was for juveniles anesthetized with 300 mg/L (Table 2).

Increasing NEOTV-T concentration reduced anesthesia induction time up to a concentration of 200 mg/L. The concentration of 300 mg/L of NEOTV-T provided an induction time similar to that of 200 mg/L (Table 2). Juveniles anesthetized with 50, 75 and 100 mg/L of NEOTV-T had similar anesthesia recovery times. From 150 mg/L onwards, increasing NEOTV-T concentration increased recovery time, reaching its greatest with 300 mg/L NEOTV-T.

When comparing EOTV-T with NEOTV-T at the same concentration, 50 and 75 mg/L of NEOTV-T caused faster anesthesia induction than did the same concentrations of EOTV-T (Table 1). No differences in induction time were observed between EOTV-T and NEOTV-T at concentrations of 100, 150, 200 and 300

mg/L. Table 2 shows the recovery times from anesthesia and the lack of differences between juveniles anesthetized with EOTV-T and NEOTV-T at concentrations 50, 75 and 100 mg/L. Juveniles anesthetized with 150, 200 and 300 mg/L of NEOTV-T had longer anesthesia recovery times than did those anesthetized with the same concentration of EOTV-T.

Experiment 2

3.3 Water quality parameters

There were no significant differences in water quality parameters (dissolved oxygen, temperature, ammonia and pH) among treatments during the 60 days of cultivation ($P > 0.05$) (Table 3).

Table 3

Water quality parameters (mean $\pm$ standard deviation) during the cultivation of juvenile *Colossoma macropomum* subjected to biometric management with and without the use of the thymol chemotype of the essential oil of *Thymus vulgaris* (EOTV-T) and its nanoemulsion (NEOTV -T).

Treatment	Temperature (°C)	OD (mg/L)	Ammonia Total (mg/L)	pH
Control	27.35 $\pm$ 0.88A	6.02 $\pm$ 0.93A	0.46 $\pm$ 0.55A	7.11 $\pm$ 0.97A
EOTV-T	27.30 $\pm$ 0.83A	6.18 $\pm$ 0.69A	0.46 $\pm$ 0.55A	6.98 $\pm$ 1.05A
NEOTV-T	27.28 $\pm$ 0.80A	5.89 $\pm$ 0.82A	0.46 $\pm$ 0.55A	6.99 $\pm$ 1.07A
Different capital letters indicate a significant difference ($P < 0.05$) between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance. Data relating to pH were classified as non-parametric and were analyzed using the Kruskal-Wallis test followed by Dunn's post-test.				

3.4 Performance

There were no significant differences for final weight, final length, weight gain, apparent feed conversion, feed consumption and survival among treatments throughout the 60 days of cultivation ($P > 0.05$) (Table 4).

Table 4

Performance parameters (mean $\pm$ standard deviation) obtained during the cultivation of juvenile *Colossoma macropomum* subjected to biometric management with and without the use of the thymol chemotype of the essential oil of *Thymus vulgaris* (EOTV-T) and its nanoemulsion (NEOTV-T).

Treatment	FW (g)	FL (cm)	WG (%)	FC (%)	FE.C (g/fish)	SUR (%)
1 at 10 days of cultivation						
Control	6.66 $\pm$ 0.80a	7.22 $\pm$ 0.24a	132.04 $\pm$ 12.34a	0.62 $\pm$ 0.05a	2.31 $\pm$ 0.18a	100.0 $\pm$ 0.0a
EOTV-T	6.38 $\pm$ 1.35a	7.15 $\pm$ 0.47a	130.33 $\pm$ 21.28a	0.66 $\pm$ 0.09a	2.30 $\pm$ 0.43a	100.0 $\pm$ 0.0a
NEOTV-T	6.61 $\pm$ 1.28a	7.08 $\pm$ 0.45a	139.17 $\pm$ 16.95a	0.59 $\pm$ 0.05a	2.24 $\pm$ 0.38a	100.0 $\pm$ 0.0a
11 at 20 days of cultivation						
Control	12.11 $\pm$ 1.47a	8.50 $\pm$ 0.35a	81.88 $\pm$ 20.5a	0.81 $\pm$ 0.05a	4.43 $\pm$ 0.70a	100.0 $\pm$ 0.0a
EOTV-T	11.33 $\pm$ 2.64a	8.24 $\pm$ 0.60a	77.40 $\pm$ 12.61a	0.80 $\pm$ 0.08a	3.97 $\pm$ 1.15a	100.0 $\pm$ 0.0a
NEOTV-T	12.11 $\pm$ 2.37a	8.28 $\pm$ 0.45a	82.88 $\pm$ 6.90a	0.74 $\pm$ 0.07a	4.03 $\pm$ 0.77a	100.0 $\pm$ 0.0a
21 at 30 days of cultivation						
Control	18.35 $\pm$ 2.23a	10.25 $\pm$ 0.35a	51.76 $\pm$ 10.01a	0.83 $\pm$ 0.10a	10.25 $\pm$ 0.35	100 $\pm$ 00a
EOTV-T	16.03 $\pm$ 3.16a	9.35 $\pm$ 0.54a	40.34 $\pm$ 10.51a	0.89 $\pm$ 0.07a	9.35 $\pm$ 0.54	100 $\pm$ 00a
NEOTV-T	16.50 $\pm$ 3.11a	9.72 $\pm$ 0.52a	34.80 $\pm$ 11.01a	1.07 $\pm$ 0.29a	9.72 $\pm$ 0.52	98.33 $\pm$ 2.89a
31 at 40 days of cultivation						
Control	26.84 $\pm$ 4.48a	11.06 $\pm$ 0.42a	45.54 $\pm$ 8.61a	0.83 $\pm$ 0.06a	7.00 $\pm$ 1.48	100.0 $\pm$ 0.0a
EOTV-T	23.10 $\pm$ 4.93a	11.08 $\pm$ 0.75a	46.18 $\pm$ 3.79a	0.87 $\pm$ 0.05a	6.13 $\pm$ 1.79	100.0 $\pm$ 0.0a
NEOTV-T	25.15 $\pm$ 5.40a	10.81 $\pm$ 0.75a	48.84 $\pm$ 5.52a	0.75 $\pm$ 0.07a	6.40 $\pm$ 1.48	100.0 $\pm$ 0.0a
41 at 50 days of cultivation						
Results followed by different letters indicate a significant difference (P < 0.05) by ANOVA followed by Tukey's test at 5%. Final weight (g) (FW), final length (cm) (FL), weight gain (WG), feed conversion (FC), feed consumption (FE.C) and survival (%) (Sur). Thymol chemotype of the essential oil of <i>Thymus vulgaris</i> (EOTV-T) and its nanoemulsion (NEOTV-T)						

Treatment	FW (g)	FL (cm)	WG (%)	FC (%)	FE.C (g/fish)	SUR (%)
1 at 10 days of cultivation						
Control	36.70 ± 7.03a	12.30 ± 0.67a	36.17 ± 4.52a	0.79 ± 0.09a	12.30 ± 0.67a	100.0 ± 0.0a
EOTV-T	30.18 ± 6.45a	11.57 ± 0.70a	30.96 ± 6.64a	0.85 ± 0.09a	11.57 ± 0.70a	100.0 ± 0.0a
NEOTV-T	34.35 ± 6.52a	11.92 ± 0.83a	40.38 ± 12.16a	0.79 ± 0.18a	11.92 ± 0.83a	100.0 ± 0.0a
51 at 60 days of cultivation						
Control	44.09 ± 10.37a	13.26 ± 1.05a	19.85 ± 2.06a	0.95 ± 0.11a	7.13 ± 2.86a	100.0 ± 0.0a
EOTV-T	35.01 ± 10.01a	12.41 ± 1.01a	15.34 ± 5.22a	1.29 ± 0.35a	5.62 ± 1.84a	100.0 ± 0.0a
NEOTV-T	41.17 ± 9.76a	13.03 ± 0.94a	19.44 ± 4.41a	1.05 ± 0.18a	6.94 ± 2.10a	100.0 ± 0.0a
Total 1 at 60 days of cultivation						
Control	44.09 ± 10.37a	13.26 ± 1.05a	1431.3 ± 260.1a	0.81 ± 0.02 a	35.53 ± 7.58a	100 ± 00a
EOTV-T	35.01 ± 10.01a	12.41 ± 1.01a	1165.0 ± 187.8a	0.88 ± 0.04 a	28.61 ± 7.62a	100 ± 00a
NEOTV-T	41.17 ± 9.76a	13.03 ± 0.94a	1381.1 ± 291.7a	0.68 ± 0.22 a	26.54 ± 11.66a	98.33 ± 2.89a
Results followed by different letters indicate a significant difference (P < 0.05) by ANOVA followed by Tukey's test at 5%. Final weight (g) (FW), final length (cm) (FL), weight gain (WG), feed conversion (FC), feed consumption (FE.C) and survival (%) (Sur). Thymol chemotype of the essential oil of <i>Thymus vulgaris</i> (EOTV-T) and its nanoemulsion (NEOTV-T)						

3.5. Hematology and blood biochemistry

There were no significant differences for hemoglobin (Fig. 3A), hematocrit (Fig. 3B), glucose (Fig. 4A), cholesterol (Fig. 4B), triglycerides (Fig. 4C), total protein (Fig. 4D), globulin (Fig. 4E), albumin (Fig. 4F), alanine aminotransferase (Fig. 4G) and alanine aminotransferase (Fig. 4H) among treatments during the 60 days of cultivation (P > 0.05).

4. Discussion

The zeta potential and average diameter of the nanoparticles present in a nanoemulsion can vary depending on the type of essential oil and the proportions of oil and emulsifier used (Zhao et al. 2018). The nanoemulsion produced here had a negative charge and a diameter of less than 150 nm, yet slight

variation was observed in average diameter and zeta potential during storage for 35 days. The stability and size of a nanoparticle in a nanoemulsion is related to the zeta potential of its surface, which leads to an electrostatic repulsion between the particles, preventing their coalescence (Guo et al.2024).

No mortality was observed during nor 24 hours after management and anesthesia procedures. However, 50 mg/L of EOTV-T and 300 mg/L of NEOTV-T exceeded the induction and recovery times, respectively, recommended by the literature. Previous studies have safely anesthetized *C. macropomum* with EOTV-T (Boaventura et al. 2022a), but there have been no studies of NEOTV-T, or any other thymol chemotype essential oil, as an anesthetic in fish. However, other authors have safely used nanoparticles (Ferreira et al. 2020) and nanoemulsions of other chemotypes of essential oils as anesthetics for fish (Kheawfu et al. 2022; Rodrigues et al. 2021; Yostawonkul et al. 2019).

Increasing the concentration of EOTV-T or NEOTV-T reduced induction time and increased recovery time. NEOTV-T was able to induce anesthesia within the range recommended by the literature (less than 2 minutes) (Keene et al. 1998) at concentrations lower than EOTV-T could. Furthermore, higher concentrations of NEOTV-T increased anesthesia recovery time when compared to the same doses of EOTV-T. The increased efficiency of essential oils in nanoparticle form is related to their greater miscibility in water and better absorption by the body (Kheawfu et al. 2017; López-Cánovas et al. 2020; Rodrigues et al. 2021). Ferreira et al. (2020) compared the use of zein nanoparticles containing eugenol (positive charge) during anesthesia of *Oreochromis niloticus* and found that the anesthesia induction time for fish anesthetized with 80 mg/L of eugenol was similar to that for animals anesthetized with 40 mg/L of nanoparticles. Kheawfu et al. (2017) compared the efficiency of clove oil and its nanoemulsion (negative charge) as an anesthetic for *O. niloticus* and also observed a shorter anesthesia induction time for animals anesthetized with the nanoemulsion. The present study found no significant difference in induction and recovery intervals using 50 mg/L of NEOTV-T and 75 mg/L of EOTV-T, and so they were selected to carry out the second experiment.

The constant use of anesthetics (50mg/L of NEOTV-T and 75 mg/L of EOTV-T) during biometrics in the cultivation of *C. macropomum* did not influence its performance, showing that these substances can be used to facilitate its management without causing economic loss to production. Several studies have been carried out in recent years to evaluate the influence of anesthetics on the physiological responses of animals (Boaventura et al. 2022a; Souza et al. 2018; Souza et al. 2019; Iversen et al. 2009; Purbosari et al. 2019). The present study, however, was the first to evaluate responses related to production parameters (performance) with the continuous use of anesthetics during animal handling. Pirhonen and Schreck. (2003) studied feed consumption of *Oncorhynchus mykiss* after handling with and without the use of anesthesia (MS-222, clove oil and CO₂) and observed the lowest feed consumption for animals anesthetized with MS- 222 and oil. Boaventura et al. (2022a) studied the influence of two chemotypes (thymol and linalool) of the essential oil of *T. vulgaris* on feed consumption of *C. macropomum* after transport and observed no difference in feed consumption between fish transported with and without the presence of the anesthetic. However, these studies only evaluated the influence of these anesthetics on

feed consumption in a short period of time (5 days), without presenting data on zootechnical performance.

No differences were observed in the analyzed hematological and biochemical parameters. The concentrations of EOTV-T and NEOTV-T used in biometric management did not cause stress or chronic metabolic or physiological changes in the juveniles. Despite being used to mitigate the effects of stress, the use of anesthetics can also induce acute stress and hematological and biochemical changes in animals (Aydın and Barbas. 2020; Souza et al. 2019; Purbosari et al. 2019). However, the influence of these anesthetics on acute responses, which are generally observed shortly after anesthesia, were not evaluated in the present study.

Hematological parameters are indicative of animal health and stress status, and may indicate the ability of a fish to adapt to new environmental conditions (Ananias et al. 2024). As there was no change in hemoglobin and hematocrit levels during the experiment, we suggest that the use of the anesthetics did not influence the oxygen transport of the studied fish during production. Increased hematocrit and hemoglobin levels are generally observed in situations of stress, physical exertion, or hypoxia exposure (Witeska et al. 2022).

When subjected to stress, an organism goes through a series of physiological changes to mobilize energy reserves to increase energy production (Boaventura et al. 2022b; Iversen et al. 2009). Increased levels of cholesterol, triglycerides and glucose may be indicative of stress, as they can be attributed to the production of cortisol (Assis et al. 2024; Godavarthy et al. 2012) and mobilization of fatty acids in the body to increase production energy (Lepic et al. 2014; Santos et al. 2021). In addition to stress-related changes, cholesterol and triglyceride levels can also provide important information about the dietary and metabolic conditions of animals (Santos et al. 2021). As no differences were observed in these parameters in the present study, we suggest that the use of anesthetics did not affect the metabolism of the juveniles, nor did it cause them stress. The enzymes ALT and AST are used as indicators of tissue damage and are also involved in the mobilization of amino acids through gluconeogenesis for the production of glucose (Boaventura et al. 2020b; Chatterjee et al. 2006). An increase in ALT and AST levels could suggest a response related to stress or tissue damage caused by the use of anesthetics. Therefore, the use of EOTV-T and NEOTV-T did not show signs of tissue damage that would alter ALT and AST levels in the body.

Furthermore, EOTV-T and NEOTV-T also did not cause changes in the levels of total plasma protein, including albumin and globulins, which could be an indication of changes in osmotic pressure, transport substances (Metcalf et al. 2007) and immunological response (Khalil et al. 2023) or intoxication to contaminants (Javed and Usmani. 2015), which in this case could be the anesthetics used.

5. Conclusions

We conclude that the nanoemulsion of the thymol chemotype of the essential oil of *Thymus vulgaris* has a diameter smaller than 100 nm for up to 22 days. The nanoemulsion induces anesthesia at

concentrations lower than the essential oil dissolved in alcohol and water. The continuous use of EOTV-T and NEOTV-T during biometric management did not influence performance and stress and can be used to facilitate animal management without causing economic losses to production. Future evaluations are necessary to increase the stability of the nanoemulsion for a longer period of time, and to evaluate its influence on acute stress and its use in larger animals.

Declarations

Author Contribution

Túlio Pacheco Boaventura: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Writing- Reviewing and Editing, Visualization. Fabio Aremil Costa dos Santos, Camila Gomes de Oliveira, Ricardo de Oliveira Correia, Sidney dos Santos Silva, Andre de Sena Souza, Imaculada de Moraes Carvalho Ananias and Nárcia Carolina Santos da Silva: Conceptualization, Methodology, Validation, Formal analysis, Investigation. Ronald Kennedy Luz and Juliano Elvis de Oliveira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Investigation, Writing – Original Draft, Writing- Reviewing and Editing, Visualization, Supervision, Project Administration, Funding Acquisition. All authors reviewed the manuscript

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Figures

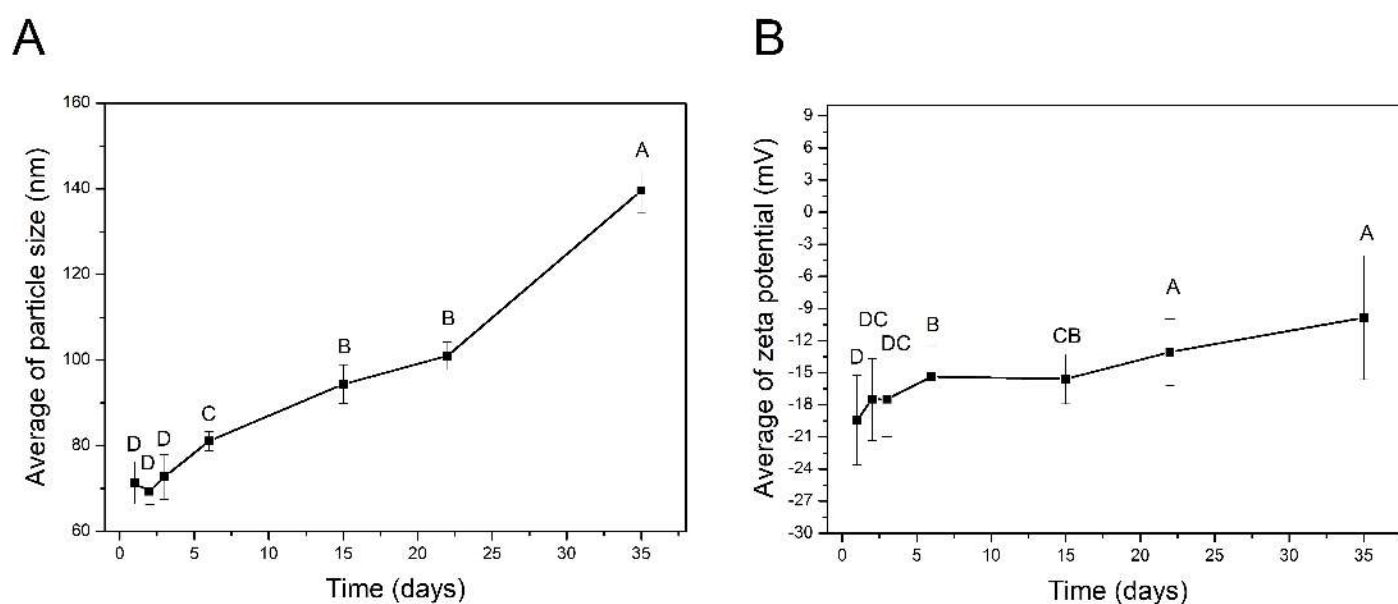


Figure 1

Particle size by DLS (A) and zeta potential (mV) (B) (mean $\pm$ standard deviation) of *Thymus vulgaris* nanoparticles analyzed on different days after nanoemulsification. Results followed by different letters differ significantly between days ($P < 0.05$). One-way ANOVA and Tukey's test were used to determine

statistical significance for particle size. Data regarding zeta potential were classified as non-parametric and so were analyzed using the Kruskal-Wallis test followed by Dunn's post-test.

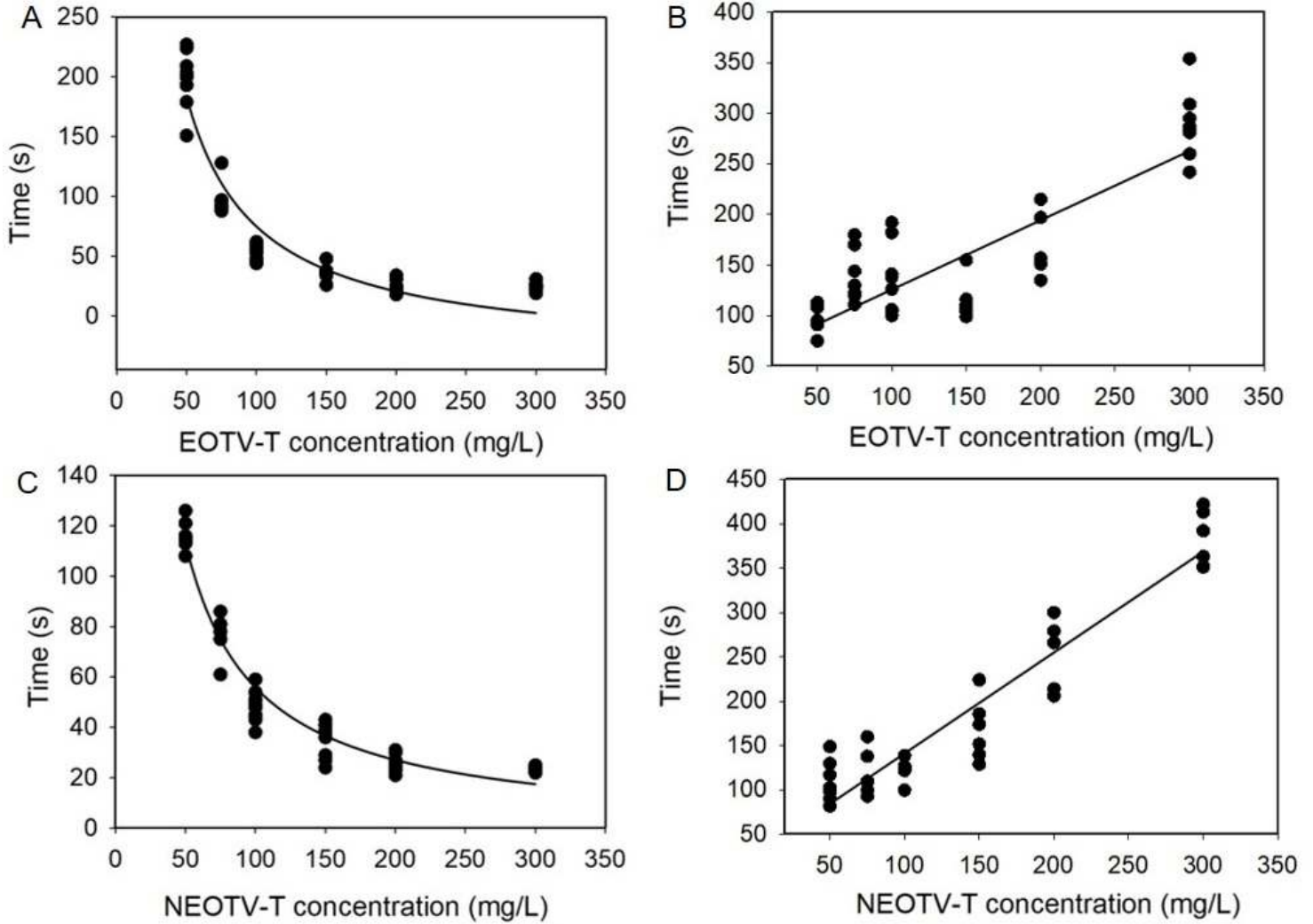


Figure 2

Anesthesia induction and recovery curve. Induction (A, B) and recovery (C, D) times for juvenile *Colossoma macropomum* (n = 10 per treatment) subjected to different concentrations of the thymol chemotype of the essential oil of *Thymus vulgaris* (EOTV-T) and its nanoemulsion (NEOTV-T). The equations for EOTV-T were fitted to the data: Induction: $Y = -33.5201 + (10845.1284/x)$ $R^2 = 0.90$ (A); Recovery $Y = 55.6857 + 0.6921 \cdot X$, $R^2 = 0.86$ (B). The equations for NEOTV-T were fitted to the data: Induction ($Y = -1.6087 + (5738.3008/x)$, $R^2 = 0.95$ (C); Recovery: ($Y = 28.8568 + 1.1323 \cdot X$, $R^2 = 0.95$ (D).

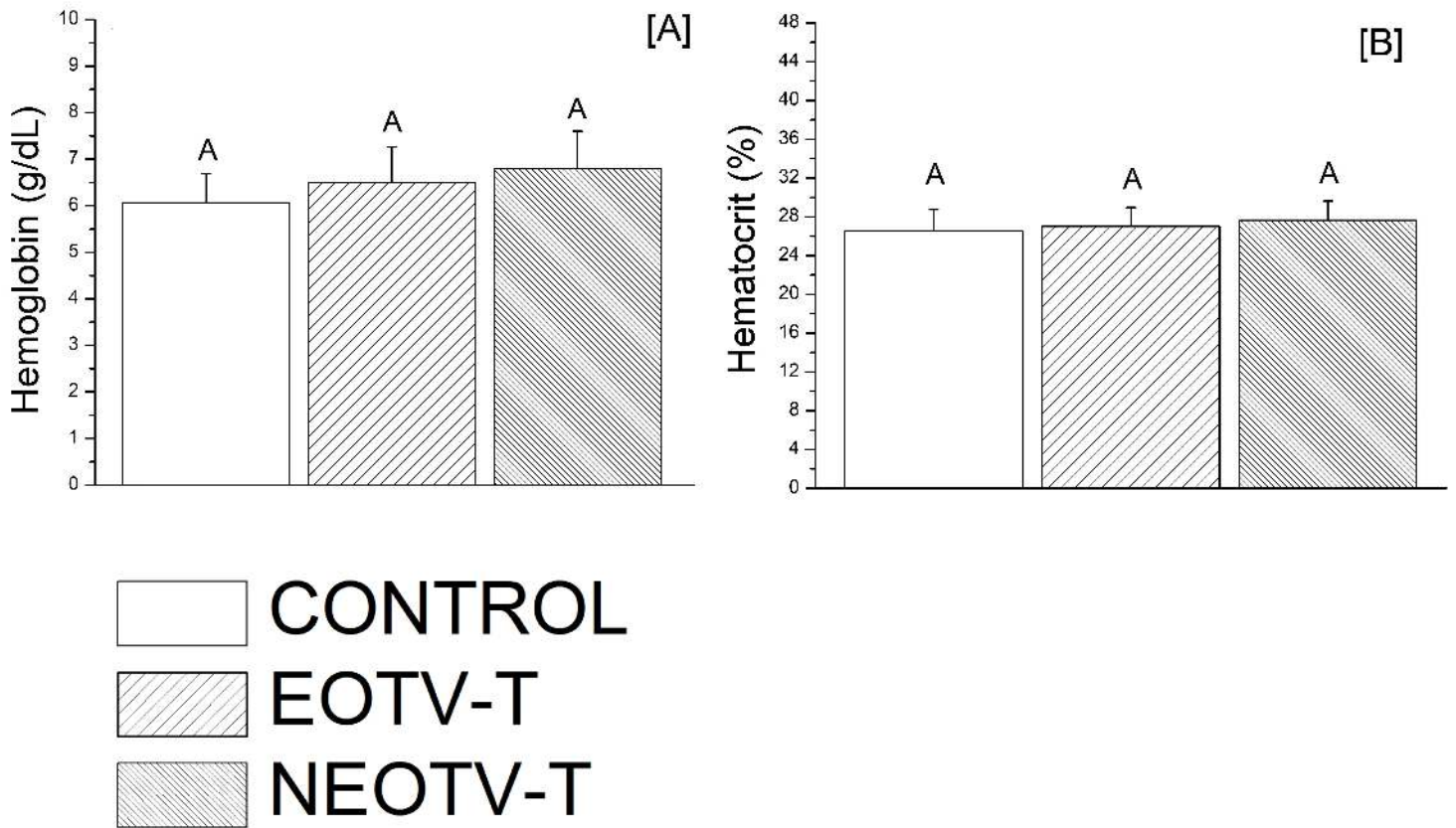


Figure 3

Hematological analyses (mean $\pm$ standard deviation) for juvenile *Colossoma macropomum* after 60 days of cultivation with subjection to biometric management with and without the use of the thymol chemotype of the essential oil of *Thymus vulgaris* of the (EOTV-T) and its nanoemulsion (NEOTV-T). Hemoglobin (A) and hematocrit (B). Results followed by different letters differ significantly between treatments ($P < 0.05$). Data relating to hematocrit were classified as non-parametric and were analyzed using the Kruskal-Wallis test followed by Dunn's post-test. Data for hemoglobin were analyzed by One-way ANOVA and Tukey's test.

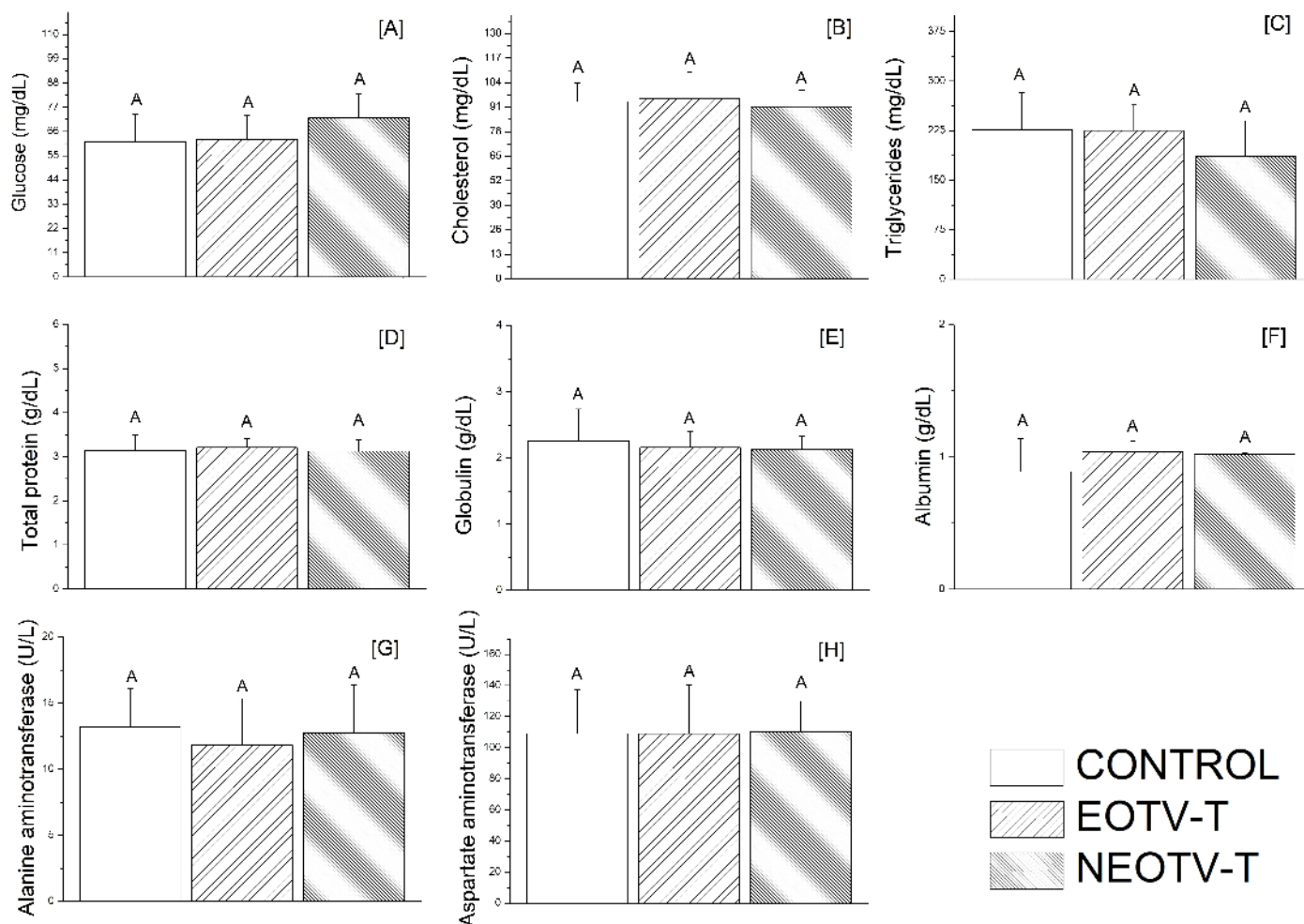


Figure 4

Biochemical and enzymatic analyses (mean $\pm$ standard deviation) for juvenile *Collossoma macropomum* after 60 days of cultivation with subjection to biometric management with and without the use of the thymol chemotype of the essential oil of *Thymus vulgaris* (EOTV-T) and its nanoemulsion (NEOTV-T). Glucose (A), cholesterol (B), triglycerides (C), total protein (D), globulin (E), albumin (F), alanine aminotransferase (G) and aspartate aminotransferase (H). Results followed by different letters differ significantly between treatments ($P < 0.05$). Data relating to albumin were classified as non-parametric and were analyzed using the Kruskal-Wallis test followed by Dunn's post-test. The remaining results were analyzed by One-way ANOVA and Tukey's test.