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Anesthetic efficacy and hemato-biochemical effects of thymol on juvenile Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

In this study, two experiments were performed to investigate the anesthetic efficiency, hematological and biochemical effects of thymol on Nile tilapia, *Oreochromis niloticus*. In the first experiment, in order to estimate thymol anesthetic efficacy, tilapia juveniles were exposed to anesthetic baths at six different concentrations of thymol (10, 20, 40, 60, 80, and 100 mg L^{-1}) and the induction and recovery times were recorded. The second experiment was performed with five treatments: CON (non-anesthetized fish), TH1 (anesthesia with 80 mg L^{-1} thymol within 1 min), TH5 (anesthesia with 30 mg L^{-1} thymol within 5 min), EU1 (anesthesia with 80 mg L^{-1} eugenol within 1 min), and EU5 (anesthesia with 30 mg L^{-1} eugenol within 5 min). The results showed that thymol at 10 mg L^{-1} concentration failed to induce anesthesia; however 20–100 mg L^{-1} concentrations induced anesthesia within 491–56.3 s. A strong negative relationship was detected between thymol concentrations and induction times ($R^2 = 0.906$). Anesthesia had no significant effects on plasma albumin, triglyceride, cholesterol, alanine aminotransferase, and the blood mean corpuscular hemoglobin levels. The anesthetized fish exhibited significant elevations in plasma cortisol, glucose, lactate, and the blood hematocrit and mean corpuscular volume; these parameters exhibited significant elevations in the fish anesthetized by 30 mg L^{-1} anesthetics, compared to the 80 mg L^{-1}. Plasma globulin, total protein, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, catalase, superoxide dismutase, and the blood hemoglobin and red blood cell significantly increased, as mean corpuscular hemoglobin concentration decreased, in the fish anesthetized by 30 mg L^{-1} anesthetics, compared to the CON fish. The anesthetized fish showed similar plasma glutathione levels, all significantly lower than the CON fish. The EU5 fish exhibited significant elevation in the plasma malondialdehyde levels, compared to the CON fish. In conclusion, thymol anesthetic efficacy was similar to eugenol in Nile tilapia weighing ~40 g at water temperature of 26 °C. Considering the physiological responses, slight but significant elevations in plasma proteins, lactate, lactate dehydrogenase, and malondialdehyde levels attest that thymol may induce less stress, hypoxia, and oxidative stress in the fish than eugenol, but further studies are needed to provide robust and clinically applicable data to support this hypothesis.

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1. Introduction

Aquaculture is one of the most important production sectors in order to meet the increasing food demand with the increasing world population. Nile tilapia, *Oreochromis niloticus*, is one of the most economically important finfish; according to the Food and Agriculture Organization, it has produced 6.5 million tons in 2018 worldwide (Hamed and Abdel-Tawwab, 2021). It is a popular fish species among farmers due to its low cost, high growth rate, environmental adaptability, and resistance to diseases and stress (Hai, 2015).

Anesthesia is an important procedure in aquaculture that affects aquaculture activities and fish welfare. Synthetic drugs such as MS-222, benzocaine, and phenoxyethanol have been used in aquaculture research and fish farming to attenuate physical damage and stress caused by weighing, handling, vaccination, spawning, blood collection, labeling, and transportation (Aydın and Barbas, 2020; Aydın and Orhan, 2021). However, because of the high cost, undesirable side effects on fish, and environmental concerns of synthetic anesthetics, several plant-based substances have attracted the attention of scientists in the recent years (Aydın and Barbas, 2020). In this regard, the number of studies carried out on plant-based anesthetics is increasing day by day due to the less harmful effects on the aquatic environment (Boaventura et al., 2020). The plant-based anesthetics are known to have many positive health benefits in fish including antimicrobial (Yostawonkul et al., 2019; Abdelkhalek et al., 2020), antioxidant (Cantanhêde et al., 2021; de Lima Silva et al., 2021), and stress-relieving and immune-promoting effects (Bandeira et al., 2017; Santos et al., 2020). Herbal anesthetics are less harmful than synthetics in terms of human and animal health, and they are already present in nature and inexpensive to manufacture (Aydın and Barbas, 2020). Thus, several recent studies have focused on the efficacy of phytochemicals as sedative and anesthetic agents in the aquatic species (Cunha et al., 2010; de Freitas Souza et al., 2018; Boaventura et al., 2020; Barbas et al., 2021).

Essential oils and their active substances with anesthetic efficacy have been previously reviewed by researchers (Aydın and Barbas, 2020). Eugenol, the active substance of clove oil, is the most commonly-used plant-based anesthetic in fish, due to its high efficacy and safety (Priborsky and Velisek, 2018; Aydın and Barbas, 2020). Other active substances, such as menthol (Kasai et al., 2014; da Silva et al., 2016; Teta and Kaiser, 2019), 1,8-cineole (Taheri Mirghaed et al., 2018), linalool (Heldwein et al., 2014; Yousefi et al., 2019), citronellal (Yousefi et al., 2019), and carvacrol (Aydın and Orhan, 2021) were recently studied as sedative and anesthetic agents in fish. These studies suggest that the plant-based active substances are effective in fish sedation and anesthesia. Compared to other fish such as common carp, *Cyprinus carpio,* and silver catfish, *Rhamdia quelen,* anesthesia studies have been less conducted on Nile tilapia. Among the active substances, only eugenol (Moreira et al., 2010; Rairat et al., 2021; Zahran et al., 2021) and menthol (Simões and Gomes, 2009; Teixeira et al., 2011) have been studied in Nile tilapia. Therefore, the efficiency of other active substances needs to be tested in this species with the help of physiological and health parameters.

Thymol (2-isopropyl-5-methylphenol), which is extracted from some medicinally important plants such as thyme, *Thymus vulgaris*, oregano, *Origanum onites*, ajwain, *Carum copticum*, honey balm, *Monarda didyma*, and *Zataria multiflora,* is a key phenolic component of the oils (Alagawany et al., 2021). Thymol has pharmacological properties including immune-promoting, stress-relieving, antioxidant, antimicrobial, and anti-inflammatory effects in fish (Ahmadifar et al., 2011; Giannenas et al., 2012; Alagawany et al., 2021). Thymol has been recently studied as an anesthetic drug in common carp (Yousefi et al., 2018), silver catfish (Bianchini et al., 2017), *Garra rufa* (Aydın and Orhan, 2021); however, there is no research about thymol anesthetic and health effects on Nile tilapia. Therefore, the objective of the present study was to investigate the effects of thymol on anesthesia of Nile tilapia juveniles, as well as to evaluate the hematological, antioxidant, and oxidative stress status of the fish exposed to thymol, compared to the most-commonly used herbal anesthetic, eugenol.

2. Materials and methods

2.1. Fish and acclimation period

This experiment was conducted after approval of the Scientific Board of Inland Water Aquatic Resources Researches Center, Gorgan, Iran. Nile tilapia juveniles were purchased from a private fish farm and transferred to the laboratory. The fish (150 individuals with average weight of 35.8 ± 1.55 g) were stocked in six 200 L fiberglass tanks for 10 days to acclimatize to the experimental conditions. The tanks were continuously aerated, and their water was replaced with clean water every day (50% renewal rate). Water temperature, pH, dissolved oxygen, and unionized ammonia levels were 26 ± 1 °C, 7.69 ± 0.49, 6.20 ± 0.88 mg L^{-1}, and 0.02 ± 0.006 mg L^{-1}, respectively. A 12 h light: 12 h dark photoperiod was maintained with fluorescent lights during the acclimation period and experiment. In the acclimation period, the fish were fed twice a day (09.00 and 16.00) with a commercial feed (Bayza Co., Shiraz, Iran; protein 32%, fat 8%) to the apparent satiation. Feeding was stopped 24 h before the experiments. At the end of the acclimation period, the fish weight was 43.60 ± 1.65 g.

2.2. Anesthetic agents

Thymol (purity ≥99%) and eugenol (purity ≥99%) were purchased from Sigma-Aldrich Corporation (St. Louis, USA) and dissolved in 96% ethanol at a ratio 1: 9 (anesthetic: ethanol) to increase water solubility before use. The mixtures were prepared prior to the start of experiments.

2.3. Experiment 1: Evaluation of anesthetic efficiency of thymol

The fish were exposed to six concentrations (10, 20, 40, 60, 80, and 100 mg L^{-1}) of thymol to record induction time of anesthesia (stage 3 and stage 4; Table 1) and recovery time (Table 1). Anesthetic chambers were 10-L plastic containers, which were aerated continuously. Six fish were caught from the 200-L tanks and placed in the anesthetic chambers, individually, and induction times were recorded using a digital timer. After the anesthesia, the fish weigh and length were recorded within 45 s; then they were placed in a 10-L plastic containers with continuous aeration for recovery. Each fish was used only once in the experiment. The fish behavioral responses to anesthesia were monitored over a 10-min period. After the experiment, the fish were monitored up to 48 h to record survival rate.

2.4. Experiment 2: Evaluation of hemato-biochemical responses to anesthesia with thymol

In this experiment, hematological and biochemical responses of Nile tilapia were assessed after exposure to short-term baths with different thymol concentrations. Moreover, eugenol was used as the reference

Table 1
Behavioral observations of different anesthesia stages (Tarkhani et al., 2017).

Stage	Exhibited behavior
0	Normal.
I	Relaxation and no response to stimuli: fish were calmed and did not respond to tactile touch.
II	Imbalance swimming: fish loss their equilibrium and show imbalance swimming.
III	Total loss of equilibrium: fish laid on lateral side, slightly depressed but regular opercular movement
IV	Deep anesthesia: slow and irregular opercular movement.
V	Death: opercular movement ceased.
Recovery	Fish regained its equilibrium and normal swimming

anesthetic, because it is the most commonly used plant-based anesthetic in aquaculture. The remaining fish in the 200-L tanks were used in this experiment. Ninety fish were selected and stocked in 15 aquaria (50-L) and kept under continuous aeration and daily water renewal rate of 50%, for 7 days to acclimatize to the experimental conditions. The fish were fed a commercial feed (as mentioned above), twice a day until satiation. After the acclimation, the fish were anesthetized and sampled. There were five treatments (each included three aquaria) in this experiment: CON (non-anesthetized fish), TH1 (anesthesia with 80 mg L^{-1} thymol within 1 min), TH5 (anesthesia with 30 mg L^{-1} thymol within 5 min), EU1 (anesthesia with 80 mg L^{-1} eugenol within 1 min), and EU5 (anesthesia with 30 mg L^{-1} eugenol within 1 min). The concentrations of thymol in this experiment were obtained from the data of the experiment 1, and the eugenol concentrations were obtained based on a preliminary test (recording the induction time of stage 4 anesthesia at 20–80 mg L^{-1} eugenol; Supplementary data). The CON fish were gently caught with a dip net, kept in a towel, immobilized by a sharp blow on the head, and sampled by caudal puncture. Two fish were sampled per aquarium and the whole procedure from catch to sample collection was executed within 60 s for each fish to ensure minimum stress. To collect the blood samples from the anesthetized fish, two fish were caught from each aquarium (six fish per treatment) and placed in an anesthetic chamber (10-L plastic containers with aeration) and sampled at the same time by two persons. The blood samples (0.8–0.9 mL) were taken from the caudal vein of the fish, using heparinized syringes (2 mL). A fraction of the sampled blood (150 μL) was placed into plastic tubes for hematological analysis. The other fraction was centrifuged (7000 rpm; 7 min; 4 °C) to obtain plasma (at least 400 μL). The collected plasma samples were stored at −70 °C for seven days before analysis.

2.5. Hematological analysis

The numbers of red blood cells (RBC) were counted using a Neubauer chamber after dilution and staining with Dacie's solution. Percentage of the hematocrit (Hct) was determined after centrifugation (13,000 rpm; 7 min), and hemoglobin (Hb) levels were measured, using a commercial kit (Zistchem Co., Tehran, Iran) according to previous studies (Blaxhall, 1972; Taheri Mirghaed et al., 2018). From these results, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to previously suggested formula (Blaxhall, 1972).

2.6. Plasma biochemical analysis

Plasma glucose, lactate, total protein, albumin, triglyceride, and cholesterol levels were measured spectrophotometrically based on, respectively, GOD-PAP, LOD-PAP, biuret, bromocresol green, GPO-PAP, and CHOD-PAP methods, using commercial kits (Pars Azmun Co., Tehran, Iran). The globulin level was calculated by the subtracting albumin from total protein. A competitive ELISA kit (IBL Co., Gesellschaft für Immunchemieund Immunbiologie, Germany) was used to measure cortisol levels according to the manufacturer protocol. Detection limit of the kit ranged 0–800 ng mL^{-1}. Inter- and intra-assay variations of the assays were 7.32% and 5.98%, respectively.

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) activities were determined kinetically using commercial kits (Pars Azmun Co., Tehran, Iran), and measured with an autoanalyzer. Plasma antioxidant parameters were measured by microplate reader (Hospitex, IEI000001, Antagynes str., LT-47164 Kaunas, Lithuania). Plasma superoxide dismutase (SOD) activity was determined based on the reduction of the cytochrome C, using a commercial kit purchased from ZellBio GmbH Company (ZellBio GmbH, Veltinerweg, Germany). Plasma catalase (CAT) activity was measured according to a previously published method (Goth, 1991) by measuring the rate of hydrogen peroxide decomposition, using a commercial kit (ZellBio GmbH, Veltinerweg, Germany). Plasma glutathione (GSH) levels were determined based on conversion of GSH to glutathione disulfide using a commercial kit (ZellBio GmbH, Veltinerweg, Germany). Levels of plasma malondialdehyde (MDA) were determined based on the reaction with thiobarbituric acid at 95 °C, using a commercial kit (ZellBio GmbH, Veltinerweg, Germany).

2.7. Statistical analysis

Normal distribution and homoscedasticity of the data were confirmed by Shapiro-Wilk and Levene tests, respectively. Comparison among the means was conducted by one-way ANOVA and Duncan test. The statistical analysis were conducted in SPSS v.22 (IBM Corp., Armonk, New York, USA), and the data obtained were presented as mean ± standard error (SE).

3. Results

3.1. Anesthesia induction and recovery times

Induction and recovery time of the fish in response to different concentrations of thymol are presented in Table 2. The fish reached the stage 3 anesthesia within 245–27.8 s, when exposed to 10–100 mg L^{-1} thymol. Thymol at 10 mg L^{-1} concentration failed to induce the stage 4 anesthesia; however 20–100 mg L^{-1} thymol concentrations induced the stage 4 anesthesia within 491–56.3 s. The shortest induction time was detected at 80 and 100 mg L^{-1} thymol concentrations ($P < 0.05$). No mortalities were observed during and at the end of the experiment 1. There were strong relationships between the time to reach stage 3 and 4 anesthesia, and thymol concentrations (Table 2). There was a significant difference in recovery time among the thymol concentrations ($P < 0.05$). The recovery time showed a declining trend when thymol concentration increased from 20 to 80 mg L^{-1}; however, 100 mg L^{-1} thymol significantly increased recovery time, compared to 80 mg L^{-1} ($P < 0.05$).

3.2. Hematological parameters

At the end of the experiment 2, hematological indices of Nile tilapia are illustrated in Fig. 1. RBC and Hb contents of TH1 and EU1 fish were similar to the CON fish, but TH5 and EU5 showed significant elevation in blood RBC and Hb concentrations. Levels of Hct and MCV significantly increased in the anesthetized fish, compared to the CON fish. TH1 and EU1 treatments presented similar Hct and MCV levels and significantly lower than TH5 and EU5. Blood MCH were similar among the treatments; however, TH5 and EU5 exhibited significantly lower blood MCHC, compared to the CON fish.

Table 2
Time (s) to reach stage 3 and 4 anesthesia and recovery of Nile tilapia, *Oreochromis niloticus* exposed to the different concentrations of thymol.

	Anesthesia time (s)		
Thymol concentrations (mg L^{-1})	Stage 3	Stage 4	Recovery time
10	245.0 ± 10.1^{e}	–	–
20	167.0 ± 6.1^{d}	491 ± 12.6^{d}	498 ± 9.65^{e}
40	116.0 ± 2.2^{c}	239 ± 13.9^{c}	467 ± 7.18^{d}
60	57.3 ± 2.9^{b}	116 ± 4.2^{b}	412 ± 5.63^{c}
80	29.0 ± 1.9^{a}	59.8 ± 3.4^{a}	301 ± 8.79^{a}
100	27.8 ± 1.4^{a}	56.3 ± 3.5^{a}	336 ± 7.42^{b}
Equation	$y = 3042\times^{-1}$	$y = 38{,}805\times^{-1.43}$	–
R^2	0.906	0.955	–

Different letters within a column indicate significant differences among the concentrations ($P < 0.05$). Data are expressed as the means ± SE ($n = 6$).

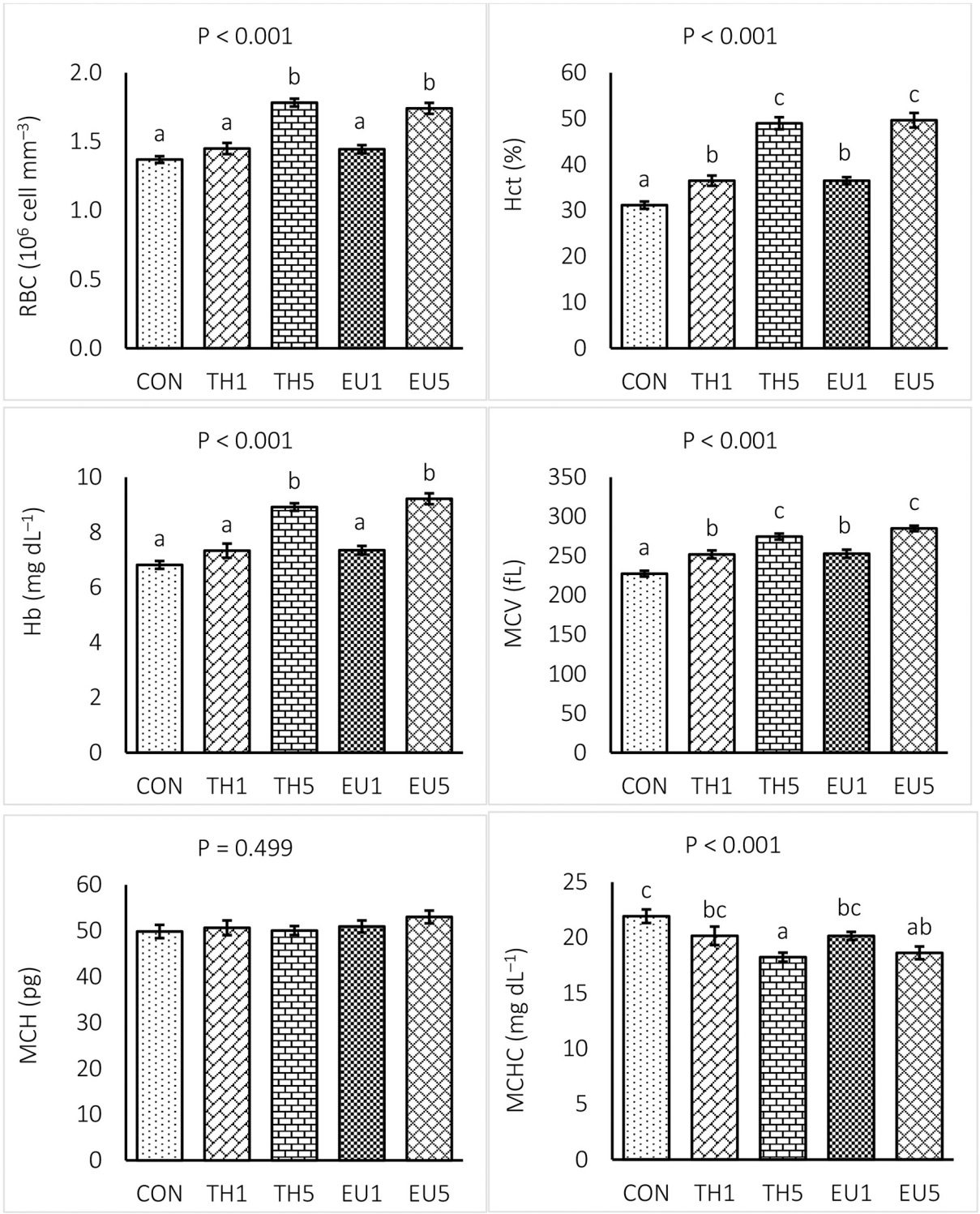

Fig. 1. Hematological parameters of Nile tilapia, *Oreochromis niloticus,* anesthetized with eugenol and thymol. Different letters above the bars indicate significant differences among the treatments (n = 6). CON: non-anesthetized fish; TH1: anesthesia with 80 mg L^{-1} thymol within 1 min; TH5: anesthesia with 30 mg L^{-1} thymol within 5 min; EU1: anesthesia with 80 mg L^{-1} eugenol within 1 min; EU5: anesthesia with 30 mg L^{-1} eugenol within 5 min.

3.3. Plasma biochemical parameters

There was no significant difference in plasma ALT activity among the treatments (Fig. 2). Plasma AST and ALP activities of TH1 and EU1 fish were similar to the CON fish, but TH5 and EU5 treatments showed significant elevation in plasma AST and ALP activities (Fig. 2).

There were no significant differences in plasma albumin, triglyceride and cholesterol levels among the treatments (Table 3). Plasma total protein and globulin levels in TH1 treatment were similar to the CON fish; however, the other anesthetized fish exhibited significant elevation in these parameters. TH5 and EU5 treatments exhibited similar plasma total protein and albumin levels; nevertheless, EU1 fish had significantly higher plasma globulin level compared to TH1 fish (Table 3).

Plasma cortisol levels significantly increased in the anesthetized fish,

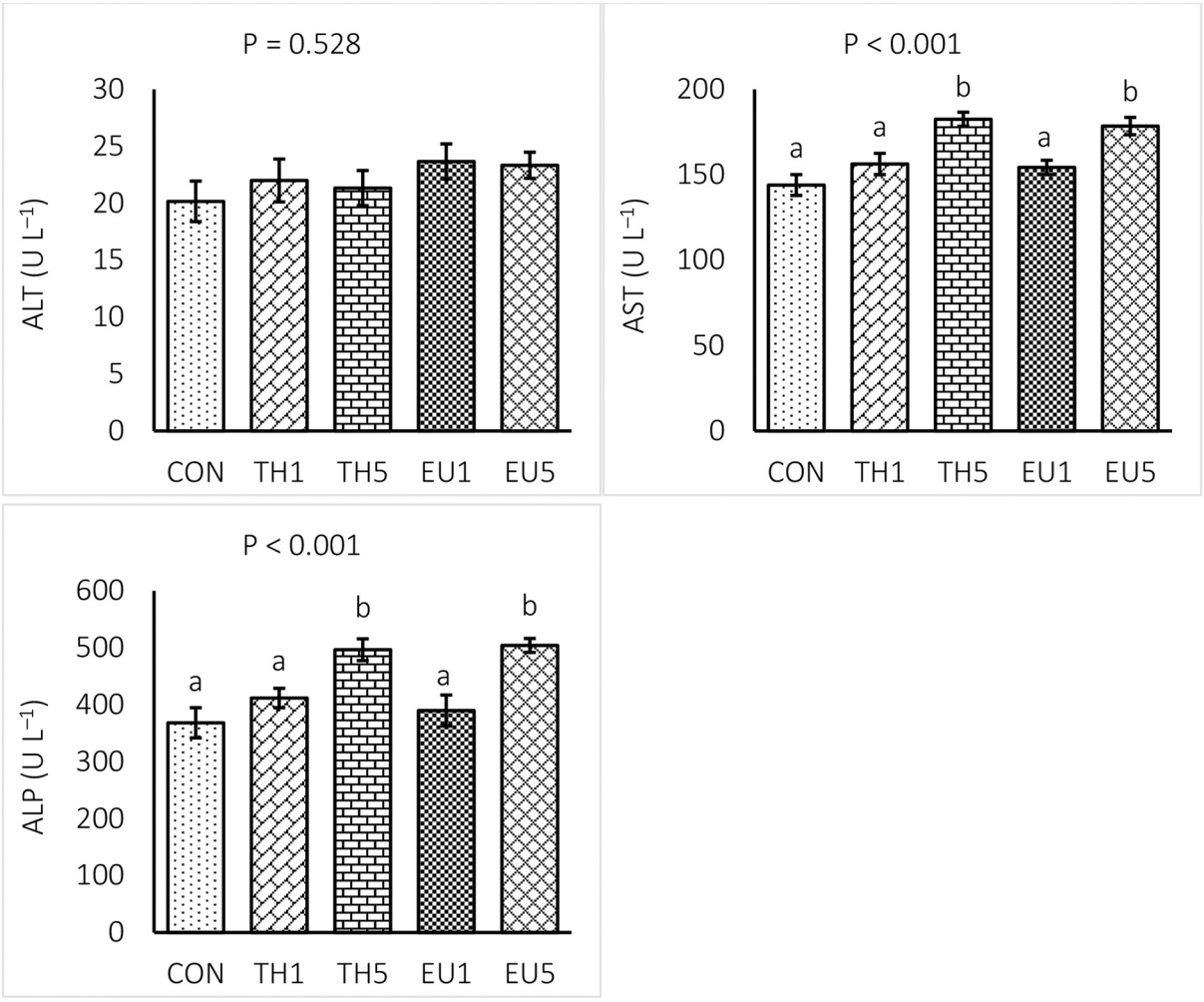

Fig. 2. Plasmatic activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in Nile tilapia, *Oreochromis niloticus,* anesthetized with eugenol and thymol. Different letters above the bars indicate significant differences among the treatments (n = 6). CON: non-anesthetized fish; TH1: anesthesia with 80 mg L^{-1} thymol within 1 min; TH5: anesthesia with 30 mg L^{-1} thymol within 5 min; EU1: anesthesia with 80 mg L^{-1} eugenol within 1 min; EU5: anesthesia with 30 mg L^{-1} eugenol within 5 min.

Table 3
Plasma biochemical parameters of Nile tilapia, *Oreochromis niloticus* anesthetized with 80 and 30 mg L^{-1} eugenol and thymol within 1 and 5 min, respectively.

	Control	Thymol		Eugenol		*P*-value
		1 min (80 mg L^{-1})	5 min (30 mg L^{-1})	1 min (80 mg L^{-1})	5 min (30 mg L^{-1})	
Total protein (g L^{-1})	31.5 ± 1.56^{a}	34.0 ± 1.36^{ab}	39.5 ± 1.60^{c}	37.8 ± 1.17^{bc}	40.5 ± 1.66^{c}	0.001
Albumin (g L^{-1})	14.4 ± 0.71	15.3 ± 0.88	15.3 ± 1.05	15.6 ± 0.72	13.9 ± 0.58	0.547
Globulin (g L^{-1})	17.1 ± 1.05^{a}	18.7 ± 1.28^{ab}	24.2 ± 0.60^{cd}	22.3 ± 1.68^{c}	26.6 ± 1.44^{d}	<0.001
Triglyceride (mg dL^{-1})	156 ± 4.29	159 ± 5.46	158 ± 7.98	158 ± 6.39	162 ± 7.04	0.968
Cholesterol (mg L^{-1})	92.2 ± 2.22	98.7 ± 2.55	93.2 ± 2.95	96.2 ± 2.18	98.8 ± 2.10	0.190

Significant differences among the treatments have been indicated by different letters with a row (mean ± SE) ($n = 6$).

compared to the CON fish ($P < 0.05$). TH1 and EU1 treatments presented similar plasma cortisol levels and significantly lower than TH5 and EU5 ($P < 0.05$) (Fig. 3). Plasma glucose levels in TH1, TH5 and EU5 treatments were significantly higher than the CON fish; TH5 and EU5 presented similar glucose levels and significantly higher than that of TH1 ($P < 0.001$) (Fig. 3). The anesthetized fish exhibited significantly higher plasma lactate levels, compared to the CON fish. TH1 and EU1 treatments presented similar plasma lactate levels, which were significantly lower than those of TH5 and EU5. The highest plasma lactate level was related to EU5 fish ($P < 0.001$) (Fig. 3). Plasma LDH activity of TH1 and EU1 fish were similar to the CON fish, but TH5 and EU5 showed significant elevation in plasma LDH activity and the highest activity was related to EU5 ($P < 0.001$) (Fig. 3).

Plasma antioxidant enzymes of Nile tilapia exposed to the thymol and eugenol concentrations within 5 min (anesthesia with 30 mg L^{-1}) and 1 min (anesthesia with 80 mg L^{-1}), and without anesthetic (CON) are presented in Fig. 4. The activities of plasma SOD and CAT, and the levels of plasma MDA and GSH showed significant differences among the treatments. Plasma SOD activities of TH1 and EU1 fish were similar to the CON fish, but TH5 and EU5 showed significant elevation in plasma SOD activity ($P < 0.001$). Plasma CAT activity significantly decreased in TH1 and EU1, but increased in TH5 and EU5, compared to the CON fish ($P < 0.001$). TH1 treatment exhibited significantly lower CAT activity, compared to EU1 treatment (Fig. 4). The anesthetized fish with both anesthetics presented similar plasma GSH levels and significantly lower than the CON fish ($P < 0.001$) (Fig. 4). Plasma MDA levels of TH1, TH5 and EU1 were similar to the CON fish; whereas, the plasma

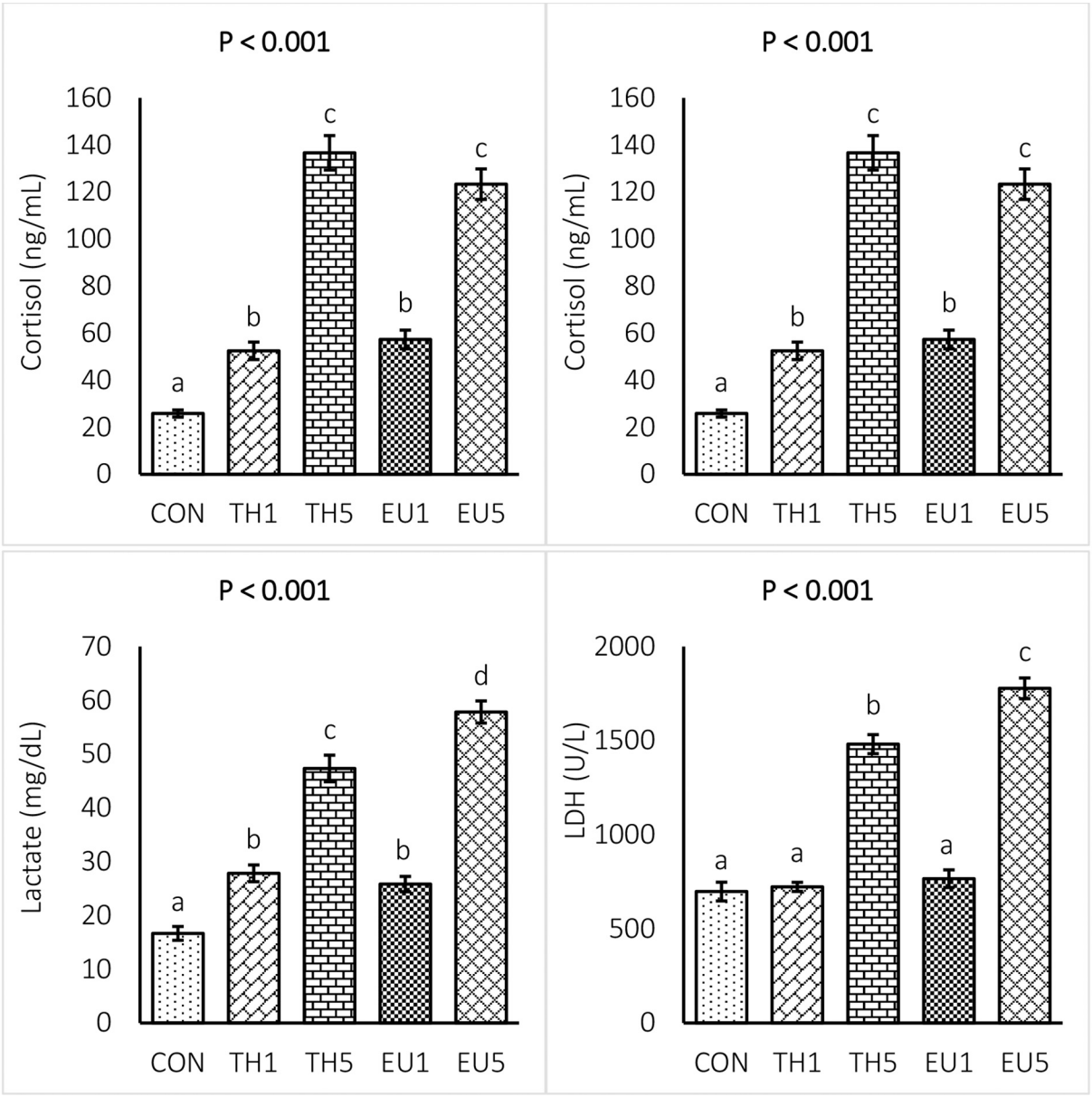

Fig. 3. Plasma cortisol, glucose, lactate, and lactate dehydrogenase (LDH) levels of Nile tilapia, *Oreochromis niloticus,* anesthetized with eugenol and thymol. Different letters above the bars indicate significant differences among the treatments (n = 6). CON: non-anesthetized fish; TH1: anesthesia with 80 mg L^{-1} thymol within 1 min; TH5: anesthesia with 30 mg L^{-1} thymol within 5 min; EU1: anesthesia with 80 mg L^{-1} eugenol within 1 min; EU5: anesthesia with 30 mg L^{-1} eugenol within 5 min.

MDA level of EU5 was significantly higher than the CON and TH5 fish (Fig. 4).

4. Discussion

In the recent years, numerous studies have been undertaken to evaluate the anesthetic effects of plant-based anesthetics in the aquatic species. There are some anesthesia studies on Nile tilapia with essential oils (Hohlenwerger et al., 2016; Netto et al., 2017; Yostawonkul et al., 2019; Ferreira et al., 2021) and active substances (Simões and Gomes, 2009; Moreira et al., 2010; Teixeira et al., 2011; Rairat et al., 2021; Zahran et al., 2021), but there is no data about the thymol efficacy as an anesthetic in this species. In the present study, thymol was found as an efficient anesthetic agent in Nile tilapia juveniles and no mortality were observed after anesthesia. Shortened induction time of anesthesia along with the increase in the thymol concentration was expected, because the fish are anesthetized when the mean effective drug concentration is met in the fish blood. Thus, a longer period of time is needed for fish anesthesia at a lower anesthetic concentration. Such data are available in Nile tilapia in response to anesthesia with eugenol, phenoxyethanol, and MS-222 (Rairat et al., 2021); however, such data for other anesthetics such as thymol should be determined in this species, yet.

According to the results, effective thymol concentration ranges 40–100 mg L^{-1}, and 80 mg L^{-1} can be used when fast anesthesia is needed, such as fish blood sampling. In general, an ideal anesthetic should induce anesthesia within 3 min, and recovery within 5 min (Marking and Meyer, 1985). According to this criterion, concentration of 80 mg L^{-1} (induction <1 min and recovery ~5 min) thymol can be suggested to be most suitable concentration for Nile tilapia anesthesia in the current study. Also, concentration of ≤10 mg L^{-1} can be used for sedation in Nile tilapia, for example during transportation. But it should be noted that these data have been obtained under the conditions of the

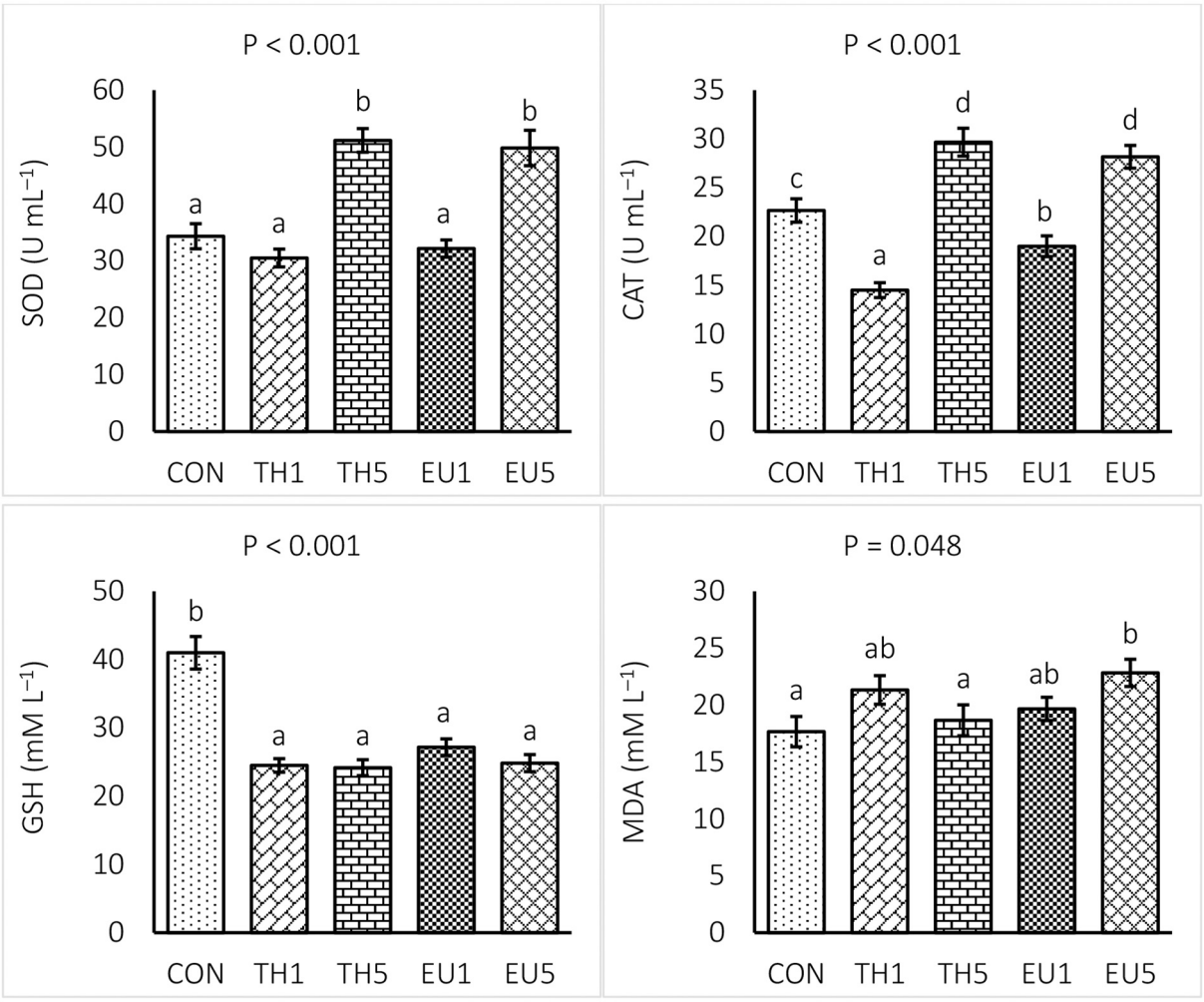

Fig. 4. Plasma antioxidant paramteres of Nile tilapia, *Oreochromis niloticus,* anesthetized with eugenol and thymol. Different letters above the bars indicate significant differences among the treatments (n = 6). CON: non-anesthetized fish; TH1: anesthesia with 80 mg L^{-1} thymol within 1 min; TH5: anesthesia with 30 mg L^{-1} thymol within 5 min; EU1: anesthesia with 80 mg L^{-1} eugenol within 1 min; EU5: anesthesia with 30 mg L^{-1} eugenol within 5 min.

present study and fish weight and water temperature, among others, are very important in anesthesia study (Rożyński et al., 2018). For example, it has been reported that eugenol induces anesthesia in Nile tilapia within 70–360 s at the concentrations of 25–300 mg L^{-1}, fish weight of 2–600 g, and water temperature of 24–28 °C (Charoendat et al., 2009; Moreira et al., 2010; Ribeiro et al., 2015; Rairat et al., 2021; Zahran et al., 2021). Comparing the present results with previous studies on thymol anesthesia in fish suggests thymol might be more efficacious to anesthetize Nile tilapia than common carp (Yousefi et al., 2018) and *G. rufa* (Aydın and Orhan, 2021). Again, differences in water temperature and fish weight must be considered among these studies, which may mask the species differences.

Hematological parameters are frequently assessed to evaluate stress in fish (Clauss et al., 2008). Plasma biochemical parameters are also widely used to assess the biochemical effects of plant-based anesthetics on fish species have been widely studied (Saccol et al., 2017; Taheri Mirghaed et al., 2018; Boaventura et al., 2020). Similar to the present results, anesthesia with other anesthetics increased RBC, Hct and Hb levels in silver catfish and beluga, *Huso huso* (Shaluei et al., 2012; Gressler et al., 2014). These increase is considered as a physiological response to increase blood oxygen capacity because of the high stress conditions in prolonged anesthesia (Taheri Mirghaed et al., 2018). In the present study, the lower anesthetic concentrations caused a significant increase in RBC, Hct, Hb, and MCV values, compared to the higher concentrations. It seems that induction time plays a role in such results, so that the maximum responses were observed at the lower thymol/eugenol concentrations. At the lower concentrations, fish need longer times to reach anesthesia, which increases stress-related parameters such as hematological parameters. Similarly, *O. mykiss* exposed to lower concentrations of 1,8 cineole (200 μL L^{-1}) exhibited elevations in the blood RBC and Hb values, compared to higher concentrations (600–800 μL L^{-1}) (Taheri Mirghaed et al., 2018).

Plasma total protein and globulin reflects the non-specific immunity statue and stress in fish, exposed to various substances (Yousefi et al., 2018; Ventura et al., 2020). Albumin is an important blood protein that is synthetized in liver and changes as a results of severe hepatic damage or hemoconcentration/hemodilution. In this study, the fish under anesthesia with both anesthetics did not show alterations in the albumin levels, which is similar to the findings of Yousefi et al. (2018), suggesting anesthesia with 30 and 80 mg L^{-1} thymol/eugenol induces no hepatic damage and/or change in the blood concentration. Plasma globulin is an indicator of stress in fish. Under a short-term stress, levels of many proteins and peptides increase as a defensive mechanism to protect the fish against the stress and immunosuppression (Caipang et al., 2009; Jia et al., 2021). In this case, heat shock protein (Roberts et al., 2010), antioxidant enzymes (Yang et al., 2017), and immune-related proteins (Caipang et al., 2009) increase, which may explain the present results. Accordingly, it seems that TH5, EU1, and EU5 treatments induced higher stress in the fish, compared to TH1. Another hypothesis may be related to hemolysis in TH5, EU1, and EU5 treatments that causes Hb

release from RBC and globulin elevation. Such results have been reported in Siberian sturgeon, *Acipenser baerii*, following anesthesia by MS-222 and eugenol (Gomulka et al., 2008). However, the degree of hemolysis might be not high in relation of erythrocytosis induced by the anesthesia stress, so that, no decrease in RBC count was observed in these treatments.

Plasma ALT, AST, and ALP are often used for monitoring and evaluation of fish tissue damage (Yousefi et al., 2021). There were no significant differences in ALT activity among the anesthetic treatments and CON fish, and this result is in accordance with earlier findings indicating that thymol and 1,8-cineole had no effect on ALT levels of *C. carpio* and *O. mykiss*, respectively (Taheri Mirghaed et al., 2018; Yousefi et al., 2018). The present results show that anesthetic exposures may have no significant effect on hepatic tissue, as ALT is found at high concentrations in the hepatocytes. However, elevation in plasma AST and ALP activities in TH5 and EU5 treatments might indicate hemolysis, as the enzymes are found at high concentrations in RBC (Gaudet et al., 1975). The results are partially supported by the levels of plasma globulin in these treatments. Due to the AST participation in glucose production from amino acids (Tejpal et al., 2009), the increased AST in TH5 and EU5 in the present study may be a stress indicator. It has been also reported that the activity of AST increases in *O. mykiss* anesthetized with low concentration of 1,8-cineole, compared to high concentrations (Taheri Mirghaed et al., 2018).

Plasma cortisol, glucose, and lactate levels are important stress responses in fish subjected to different environmental conditions, so they are valuable parameters in anesthesia studies (Teixeira et al., 2017; Taheri Mirghaed et al., 2018; Ferreira et al., 2021). During anesthesia, fish experience hypoxia due to hypoventilation and such a stress causes cortisol and glucose elevation (Sneddon, 2012); moreover, hypoxia leads to anaerobic glucose metabolism and lactate formation (van Ginneken et al., 2004). Excess lactate is converted by LDH to pyruvate and is used for glucose production; therefore, hypoxia increases LDH activity. In this study, these parameters increased in the anesthetized fish compared to the CON fish. The results are in line with previous studies, demonstrating that anesthesia induces stress, intrinsically; for example, stress responses due to anesthesia with different anesthetics were reported in beluga (Shaluei et al., 2012), pacman catfish, *Lophiosilurus alexandri* (Boaventura et al., 2020), and Nile tilapia (Teixeira et al., 2017). In the present study, cortisol, glucose, lactate, and LDH levels of fish exposed to the low concentration (30 mg L^{-1}) of thymol and eugenol significantly increased compared to the high anesthetic concentration (80 mg L^{-1}). This might be due to shorter time of anesthesia that induced lower stress in the fish. Similar to these findings, Auperin et al. (1997), Shaluei et al. (2012), and Taheri Mirghaed et al. (2018) found higher stress responses in the fish anesthetized by lower concentrations of anesthetics, compared to higher concentrations.

Anesthetic exposure affects the blood oxygen levels of fish, hence changing the antioxidant responses (Gressler et al., 2014). The present results demonstrated that the fish exposed to oxidative conditions after anesthesia with both anesthetics/concentrations, as the levels of GSH decreased in all treatments. GSH is a powerful antioxidant molecule that has radical scavenging activity and acts as a coenzyme in the antioxidant system (Galano and Alvarez-Idaboy, 2011; Higuchi, 2014). During the oxidative conditions, GSH neutralizes free radicals and is converted to glutathione disulfide; thus, the levels of GSH decrease in the fish body (Galano and Alvarez-Idaboy, 2011). However, such an antioxidant activity seems not to be enough to prevent lipid peroxidation in EU5, as plasma MDA in this treatment exhibited a significant elevation. MDA is a product of lipid peroxidation and a very toxic molecules (Yousefi et al., 2019). Combining these results with SOD and CAT data implies the lower anesthetic concentrations has induced higher oxidative conditions, compared to the higher anesthetic concentrations. These enzymes are the first antioxidant enzymes that acts under oxidative conditions to prevent oxidative stress (Yousefi et al., 2020); thus elevated level of these enzymes is a sign of oxidative conditions. The present results are partially in line with previous researches. For example, common carp anesthetized by eugenol exhibited higher plasma MDA levels, compared to the fish anesthetized by thymol (Yousefi et al., 2018). Anesthesia with cineole decreased hepatic GSH concentration in common carp; moreover, lower concentrations of the anesthetic induced higher hepatic SOD and CAT activity, compared to higher concentrations (Hoseini et al., 2019).

In conclusion, based on the induction and recovery times, thymol exhibits anesthetic effects on Nile tilapia at the concentrations of 20–100 mg L^{-1}, however, when blood parameters are taken into account, 80 mg L^{-1} of thymol and eugenol can be used for aquaculture activities and rapid anesthesia. It is also concluded that higher anesthetic concentrations may have lower detrimental effects on the fish tissue health. Based on the tested parameters, thymol and eugenol induced similar effects on the fish physiology, except a narrow but significant increase in plasma lactate, LDH, and MDA levels, suggesting 30 mg L^{-1} thymol may induce less anaerobic and oxidative conditions, compared to 30 mg L^{-1} eugenol. Whether such differences are important from the pathophysiological point of view needs further evaluations.

Author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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