

Essential oils from leaves of *Pilocarpus pennatifolius* and *Cordia verbenacea* in silver catfish (*Rhamdia quelen*): cardiovascular aspects and participation of the GABAergic system in the sedative and anesthetics effects



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This study evaluated the heart rate of silver catfish (*Rhamdia quelen*) exposed to a sedative concentration of *Cordia verbenacea* (CVOL) and *Pilocarpus pennatifolius* (PPOL) leaves essential oils (EOs) using ultrasound device with color doppler. Furthermore, it aimed to establish the possible involvement of the GABAergic receptor complex benzodiazepine site (BDZ) in their sedative and/or anesthetic effects using juvenile silver catfish as experimental model. Fish were transferred to baths with 150 μ M diazepam [DZP], DZP (150 μ M) in association with EOs sedative and anesthetic concentrations, respectively, 20 and 300 mg L⁻¹ of CVOL or PPOL, and the EOs alone. Juveniles were exposed to bath containing flumazenil [FLU] (5 μ M) or water to recover. DZP showed a positive interaction with the EOs to achieve sedation and anesthesia. Fish exposed to DZP, DZP in association with EOs and EOs alone showed a higher recovery score when exposed to FLU, suggesting the participation of the benzodiazepine site of GABA_A receptor complex in the effect of CVOL and PPOL. The major component of CVOL was α -pinene (34.8%) and of PPOL was 2-undecanone (57.2%). Both EOs showed sedative effect at 20 mg L⁻¹ and PPOL induced anesthesia at 300 mg L⁻¹, without adverse effects.

Keywords: Baleeira weed, Benzodiazepine site, Glucose, Heart rate, Jaborandi, Stress.

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Este estudo avaliou a frequência cardíaca de jundiás (*Rhamdia quelen*) expostos a uma concentração sedativa de óleos essenciais (OEs) de folhas de *Cordia verbenacea* (CVOL) e *Pilocarpus pennatifolius* (PPOL) utilizando um dispositivo de ecografia com doppler colorido. Além disso, objetivou-se estabelecer o possível envolvimento do sítio benzodiazepínico do complexo receptor GABAérgico (BDZ) nos seus efeitos sedativos e/ou anestésicos, utilizando juvenis de jundiá como modelo experimental. Os peixes foram transferidos para banhos com 150 μM de diazepam [DZP], DZP (150 μM) em associação com OEs nas concentrações sedativa e anestésica, respectivamente, 20 e 300 mg L^{-1} de CVOL ou PPOL, e os OEs isoladamente. Os juvenis foram expostos a banhos contendo flumazenil [FLU] (5 μM) ou água para a recuperação. O DZP mostrou uma interação positiva com os OEs para atingir a sedação e anestesia. Os peixes expostos a DZP, DZP em associação com OEs e OEs isolados apresentaram uma maior pontuação de recuperação quando expostos ao FLU, sugerindo a participação do sítio benzodiazepínico do complexo receptor GABA_A no efeito de CVOL e PPOL. O principal componente do CVOL foi o α -pineno (34,8%) e do PPOL foi a 2-undecanona (57,2%). Ambos os OEs apresentaram efeito sedativo na dose de 20 mg L^{-1} e o PPOL induziu anestesia na dose de 300 mg L^{-1} , sem efeitos adversos.

Palavras-chave: Erva-baleeira, Estresse, Frequência cardíaca, Glicose, Jaborandi, Sítio benzodiazepínico.

INTRODUCTION

Several synthetic drugs are used in global aquaculture to induce depression of the central nervous system (CNS), especially to lead to stages of sedation and anesthesia. Those commonly used to minimize stressful effects are mainly tricaine methanesulfonate (MS-222) and benzocaine (Mattson, Ripple, 1989; Gomes *et al.*, 2001). However, they may induce undesirable adverse effects, such as hypoxia, loss of mucus, tissue irritation, among others (Sneddon, 2012; Zahl *et al.*, 2012; Barbas *et al.*, 2017). Therefore, agents of natural origin, such as essential oils (EOs), can be an excellent alternative to synthetic drugs, because they have similar or greater efficacy and have lower toxicity rates for fish (Okamoto *et al.*, 2009; Souza *et al.*, 2019). However, some physiological parameters need to be evaluated when using these sedatives and/or anesthetics, as they can be stressful for fish.

Primary stress induces a significant increase in circulating concentrations of catecholamines and corticosteroids, which act to stimulate glycogen hydrolysis. As a result, there is an increase in blood glucose levels and heart rate (*hr*) (Jerez-Cepa, Ruiz-Jarabo, 2021). Secondary responses occur through an increase in the levels of these hormones (Barton, 2002; Maricchiolo, Genovese, 2011). When the stressor persists, tertiary responses are observed, resulting in severe damage to the fish well-being and health (Maricchiolo, Genovese, 2011; Robinson *et al.*, 2019; Sutherland *et al.*, 2019; Jerez-Cepa; Ruiz-Jarabo, 2021).

It is extremely important to know the mechanism of action of sedative and/or anesthetic agents, especially to predict important aspects regarding sedation and/or anesthesia and also to determine possible antagonists in case of need to reverse the drug effect for some specific reason, such as an administration error. In this context, many anesthetics exert their effects through modulation of the gamma-aminobutyric acid (GABA) receptor complex, the main CNS inhibition neurotransmitter (Sieghart, 2006). Activation for synaptic release of GABA occurs through ionotropic (GABA_A and GABA_C) and metabotropic (GABA_B) receptors (Tanelian *et al.*, 1993; Alexander *et al.*, 2008). Expressions of this system have already been observed in several vertebrates, with functional GABAergic evidence described in the brain of fish (Kim *et al.*, 2004; Delgado, Schmachtenberg, 2008; Garlet *et al.*, 2019a). Benzodiazepines (BDZ) have high affinity for a specific site on the GABA_A receptor, and through its binding, the pharmacological action occurs through allosteric modulation (Heldwein *et al.*, 2012). Diazepam (DZP/BDZ agonist) and flumazenil (FLU/imidazo-benzodiazepine), an antagonist of the central effects of BDZ by competition for the BDZ site (Darragh *et al.*, 1982) are widely used to establish possible positive interaction effects and mechanism of action of EOs, respectively (Heldwein *et al.*, 2012; Silva *et al.*, 2012; dos Santos *et al.*, 2017). Previous work suggested possible GABAergic modulation through GABA_A receptors for some EOs, such as those of *Ocimum gratissimum* and *Lippia alba* in silver catfish, *Rhamdia quelen* (Quoy & Gaimard, 1824) (Heldwein *et al.*, 2012; Silva *et al.*, 2012).

The EOs of the plants *Cordia verbenacea* (CVOL) (Boraginaceae) and *Pilocarpus pennatifolius* (PPOL) (Rutaceae), native to South America (Bayeux *et al.*, 2002; Carmo *et al.*, 2018), need to have their mechanism of action elucidated, as both showed promising sedative and/or anesthetic effects in Nile tilapia, *Oreochromis niloticus* (Fortes *et al.*, 2024b). *Cordia verbenacea* is popularly known as “erva-baleeira” (Michielin *et al.*, 2009; Faria *et al.*, 2023) and is found along the Brazilian coast (Dutra *et al.*, 2016), Atlantic Forest, Amazon, and Pampa (Bayeux *et al.*, 2002; Melo *et al.*, 2021; Faria *et al.*, 2023). It is worth highlighting that CVOL is extracted by Brazilian companies that produce and sell herbal medicines. *Pilocarpus pennatifolius* is popularly known as “jaborandi” or “canela-de-agouti” (Souza *et al.*, 2003; Santos, Moreno, 2004). It has a latitudinal distribution (Sawaya *et al.*, 2011) and occurs in several Brazilian states (Souza *et al.*, 2003). Therefore, this study aimed to evaluate whether the sedative and anesthetic effects of CVOL and PPOL would involve the benzodiazepine site of the GABA_A receptor. To this goal, it was assessed whether there would be a positive interaction when these EOs were associated with DZP and the reversal of the effect with the use of FLU. Blood glucose and *hr* were also determined in silver catfish exposed to these EOs, aiming to suggest a possible safe use of sedative and/or anesthetic concentrations in this species.

MATERIAL AND METHODS

Chemicals. The synthetic drugs BDZ agonist diazepam (DZP, injectable solution 5 mg mL⁻¹) and antagonist flumazenil (FLU, injectable solution 0.1 mg mL⁻¹), were purchased from Cristália Produtos Químicos e Farmacêuticos LTDA, São Paulo, Brazil.

Plant material, extraction, and chemical analysis of natural anesthetics. Leaves of *Pilocarpus pennatifolius* were collected in Santa Maria, Rio Grande do Sul State, Brazil (29°11'52"S 53°16.8'56"W). The identification of the species was made by Prof. Dr. Frederico Dimas Fleig, from the Department of Forestry Sciences at the UFSM. Voucher material was deposited in the herbarium of the Department of Biology, under registration number SMDB n° 23007. Fresh leaves of *P. pennatifolius* were used to obtain the EO (PPOL). The EO of fresh leaves of *Cordia verbenacea*, CVOL, was purchased commercially from Laszlo Aromatologia Eireli (Brazil). The extraction of PPOL was carried out by hydrodistillation for three hours, in triplicate, in modified Clevenger apparatus (European Pharmacopoeia, 2007). The PPOL was sealed and stored in an amber glass bottle at -4°C, and both EOs had a qualitative analysis of the composition and percentage of components carried out by gas chromatography in an Agilent 7890A hyphenated system, equipped with 5975C series mass selective detector and by flame ionization detector (FID), respectively. The parameters for analysis were DB5-MS fused silica capillary column (Film thickness 0.25 µm, 5% phenylmethylsiloxane, 30 m x 0.25 mm); Carrier gas 1 mL min⁻¹, He; Split input injection mode (1:50); Oven heating program (40°C), for 4 min/Ti, 40–320 °C at 4 °C min⁻¹; 250 °C injector, detector and interface temperature. The constituents were identified by comparing the fragmentation patterns of the mass spectra and the Kovats retention indices [KI], determined through a calibration curve of a homologous series of n-alkanes (C8–C40), with data from literature and the equipment library (NIST, 2008, 2024; Adams *et al.*, 2011; Silva *et al.*, 2015; Garlet *et al.*, 2019b).

Animal model (*Rhamdia quelen*, voucher number Universidade Federal do Rio Grande do Sul, UFRGS 29744) and maintenance. The fish were purchased commercially from a fish farm near Santa Maria and transported to the Fish Physiology Laboratory at the UFSM. The fish were acclimatized for one week, kept protected from light, at 23 °C, with constant aeration in 250 L tanks, fed three times a day (8, 12 and 19 h) until satiety with commercial feed (32% CP, Supra Water Line | Alisul Alimentos S.A.® Brazil). Every day, 30 min after feeding, feces and food debris were removed, and 10% of the water in the tanks was replaced. Dissolved oxygen levels (6.64 ± 0.22 mg L⁻¹) and temperature (23.0 ± 1.15 °C) were measured daily with an YSI55 oximeter (Xylem Inc, EUA), pH (7.49 ± 0.26) with a pH meter (DMPH- 2 - Digimed, SP, Brazil).

Assessment of the participation of the benzodiazepine site in the observed effect

Sedative and/or anesthetic induction. Possible central nervous depression was evaluated in juvenile silver catfish (4.98 ± 0.83 g and 6.79 ± 0.49 cm) exposed to the experimental groups (Tab. 1). The EOs were diluted previously in 95% ethanol (1:10). The anesthetic and/or sedative inducing potential and recovery was evaluated individually in 12 h-fasted juveniles in aquariums (11.5 cm high x 12.5 cm wide x 17.5 cm long) containing 1 L water with aeration according to the stages described by Gomes *et al.* (2011) with some adaptations (n = 6 juveniles): S2 (Deep sedation, without reaction to external stimuli); S3a (Partial loss of balance, the fish begin to swim sideways); S3b (Total loss of balance, the fish do not swim, but there is a

response to pressure in the caudal peduncle and the animals remain at the bottom of the aquarium); S4 (Anesthesia and loss of reflexes, with zero response to stimuli in the caudal peduncle). Thus, when the fish reached the S4 stage or after a maximum time of 30 min, they were transferred to recovery aquaria, which contained water or FLU (5 µM), and oxygenation (Heldwein *et al.*, 2012). Each juvenile was used only once, and the induction and recovery times were measured using a digital stopwatch. The control group, containing only ethanol, did not show effects of CNS depression (Heldwein *et al.*, 2012; dos Santos *et al.*, 2017).

Sedative and/or anesthetic recovery: water versus flumazenil. Based on the evaluations and observations of the induction times of the effect on the CNS, the fish were placed in a recovery aquarium with or in the absence (n = 3) of FLU (5 µM) (Heldwein *et al.*, 2012). This was to determine the possible involvement of the GABAergic system BDZ site in the observed effect (sedation or anesthesia) on the fish, with both aquariums containing water (1 L) and oxygenation. Thus, for both groups, the juveniles' recovery behavior was scored after 1, 5, 10, 15, and 20 min, adapted from Heldwein *et al.* (2012): 0 (No sign of recovery); 0.5 (Reaction after stimulation of the caudal peduncle); 1 (No posture, but showing first sign of recovery); 1.5 (Swam irregularly and then stopped); 2 (No reflexes after external stimulus, but normal swimming); 2.5 (No reflexes after external stimulus, remaining still after swimming normally); 3 (Reflexes after external stimuli and normal swimming). At the end of 20 min, the score was added up for each fish, with 0.5 being added to this sum when agitation behavior was observed. In each observation, the fish was subjected to an external stimulus with a glass rod at the bottom of the aquarium, as these tend to remain still when not exposed to external stimuli.

TABLE 1 | Acronyms and concentrations used in the study of ethanol, diazepam (DZP), DZP associated with sedative and anesthetic concentrations of essential oils (EOs) of *Cordia verbenacea* (CVOL) or *Pilocarpus pennatifolius* (PPOL) and the sedative or anesthetic concentrations of these EOs in the absence of DZP. (+) Association of synthetic drug and anesthetic and/or sedative agents (n = 6 each group).

Experimental groups	Acronyms	
	CVOL	PPOL
Ethanol (3000 µL L ⁻¹)	Ethanol	
Diazepam (150 µM)	DZP	
DZP (150 µM) + Sedative (20 mg L ⁻¹)	C1	P1
DZP (150 µM) + Anesthetic (300 mg L ⁻¹)	C2	P2
Sedative (20 mg L ⁻¹)	C3	P3
Anesthetic (300 mg L ⁻¹)	C4	P4

Determination of blood glucose levels. Blood collect was performed in the caudal region (caudal vein) of the juveniles with a sterile hypodermic needle and syringe (1mL/CC, U100, 0.45x13, 26Gx1/2”) 30 min after the start of recovery. Fish were contained with a damp cloth, covering the cranial part of the animal (eyes). Glucose was determined using a digital glucometer (G-TECH Free¹®, South Korea) and analytical strips (G-TECH Free¹®, South Korea). Measurements were carried out on all fish exposed to different concentrations (Tab. 1) and also on six animals that were not subjected to any previously described protocol (basal group).

Determination of heart rate (HR). Fish (421.8 ± 78.3 g and 35.3 ± 3.0 cm) were exposed to 20 mg L⁻¹ of CVOL (C3), 20 mg L⁻¹ of PPOL (P3), eugenol (50 mg L⁻¹, positive control) and another group without any substance immersed in the water (negative control) (n = 8 each group). When sedation (S2) was confirmed, the fish was removed from the aquarium and contained in a damp cloth (cranioventral position), to assess HR using an ultrasound device with color doppler (Mindray® Z6 Vet Gold). Heartbeats were counted with a digital stopwatch for 15 sec and the number was multiplied by four to obtain the individual's heart rate. After the treatments, the fish were placed in aquariums with 10 L of water and aeration for full recovery.

Statistical analysis. Levene's test was performed to evaluate the homoscedasticity of the data. The data are presented as mean $\pm$ standard deviation, with the significance level considered 95% ($p < 0.05$). Recovery scores were analyzed by One-Way ANOVA, followed by Tukey's test. The Kruskal-Wallis test, for non-parametric data (anesthetic induction, *hr*, glucose), followed by the Dunn test, were used to compare the other data, using the Prism version 9.0® software.

RESULTS

Chemical composition of EOs and extraction yield. The identification of the PPOL composition was 99.8%, with 2-Undecanone (57.2%) and 2-Tridecanone (28.3%) as major components. The average yield of PPOL was $0.1 \pm 0.007\%$. The major compound of CVOL was α -Pinene (34.8%) (Tab. 2).

Assessment of the participation of the benzodiazepine site in the observed effect

Sedative and/or anesthetic induction. DZP led silver catfish to stages S2, S3a, and S3b, but did not anesthetize the fish at the tested concentration. The times for DZP induce S2 and S3a are significantly higher than those of the association DZP and anesthetic concentration of CVOL (Figs. 1A–C). The fish exposed to 20 mg L⁻¹ CVOL (C3) reached only S2 and the time to reach this stage was higher than the combination DZP and anesthetic concentration of CVOL and the anesthetic concentration of CVOL alone (Fig 1). The combination DZP and anesthetic concentration of CVOL was faster to induce S3 and S4 than the anesthetic concentration of CVOL alone (Figs. 1C, D).

Rhamdia quelen juveniles exposed to 20 mg L⁻¹ PPOL alone (P3) reached only stage S2, but in combination with DZP (P1) reached up to S3b (Fig. 2). Fish submitted to

both concentrations of PPOL with DZP (P1 and P2) induced stage S2 faster than P3, and those exposed to P2 reached stage S3b faster than fish exposed to P1 and P4. Fish exposed to P2 reached S4 faster than P4 (Fig. 2).

Sedative and/or anesthetic recovery: water versus flumazenil. The recovery scores of juveniles exposed to both EOs alone or in association with DZP were higher in those that recovered in FLU than those that recovered in water (Fig. 3). Furthermore, fish exposed to C2 placed to recover in water (100%) reached the S5 stage. After 6 h of completing observations of the recovery of juveniles exposed to C2, no interest in the diet provided was observed. After 24 and 48 h, all juveniles fed, and no additional mortality was observed.

Blood glucose levels. Blood glucose levels were higher in fish exposed to C2 than basal group and those exposed to ethanol, while juveniles exposed to C4 presented higher levels than basal group (Fig. 4A). The C4 group showed higher blood glucose levels than the C3 group. In the second experiment, exposure to DZP led to higher blood glucose levels than basal group (Fig. 4B).

Cardiovascular effects: hr. Sedation (S2) with eugenol took longer than with C3. Exposure to eugenol increased heart rate, while both C3 and P3 did not change it compared to basal group (Fig. 5).

TABLE 2 | Chemical composition of the essential oils of *Cordia verbenacea* (CVOL) and *Pilocarpus pennatifolius* (PPOL). Subtitle: ^aRI = Retention index; ^bExperimental; ^cLiterature Adams (2011) and NIST (2024).

RI ^a E ^b	RI ^a L ^c	Compound	%	
			CVOL	PPOL
929	939	-pinene	34.8	-
1027	1028	Limonene	1.3	-
1292	1291	2-Undecanone	-	57.2
1301	1303	2-Undecanol	-	1.5
1388	1392	Elemene	2.7	-
1388	1387	Damascone	-	1.3
1453	1452	α-Humulene	3.8	-
1479	1480	Germacrene D	-	10.4
1494	1494	2-Tridecanone	-	28.3
1504	1504	Undecenol acetate	-	0.5
1574	1571	Spathulenol	2.8	0.6
Identified components			45.4	99.8
Unidentified components			54.6	0.2

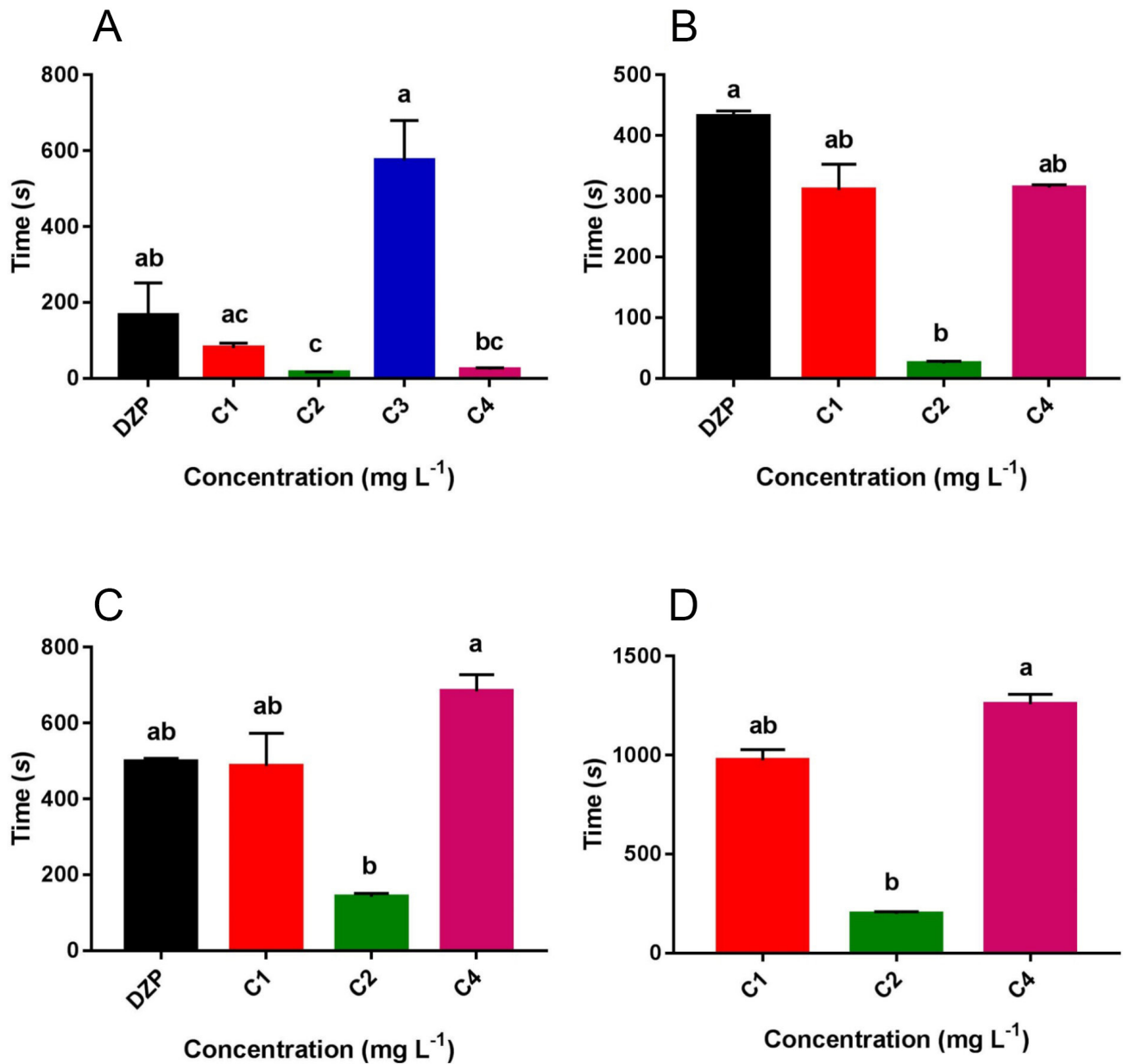


FIGURE 1 | Times to juveniles of *Rhamdia quelen* reach the stages S2 (A), S3a (B), S3b (C) and S4 (D) in response to diazepam (DZP) 150 μ M, DZP (150 μ M) associated to the sedative (C1) or anesthetic (C2) concentrations of *Cordia verbenacea* essential oil (CVOL), and sedative (C3) and anesthetic (C4) concentrations of CVOL in the absence of DZP (n = 6). C1: DZP 150 μ M + CVOL 20 mg L⁻¹; C2: DZP 150 μ M + CVOL 300 mg L⁻¹; C3: CVOL 20 mg L⁻¹; C4: CVOL 300 mg L⁻¹.

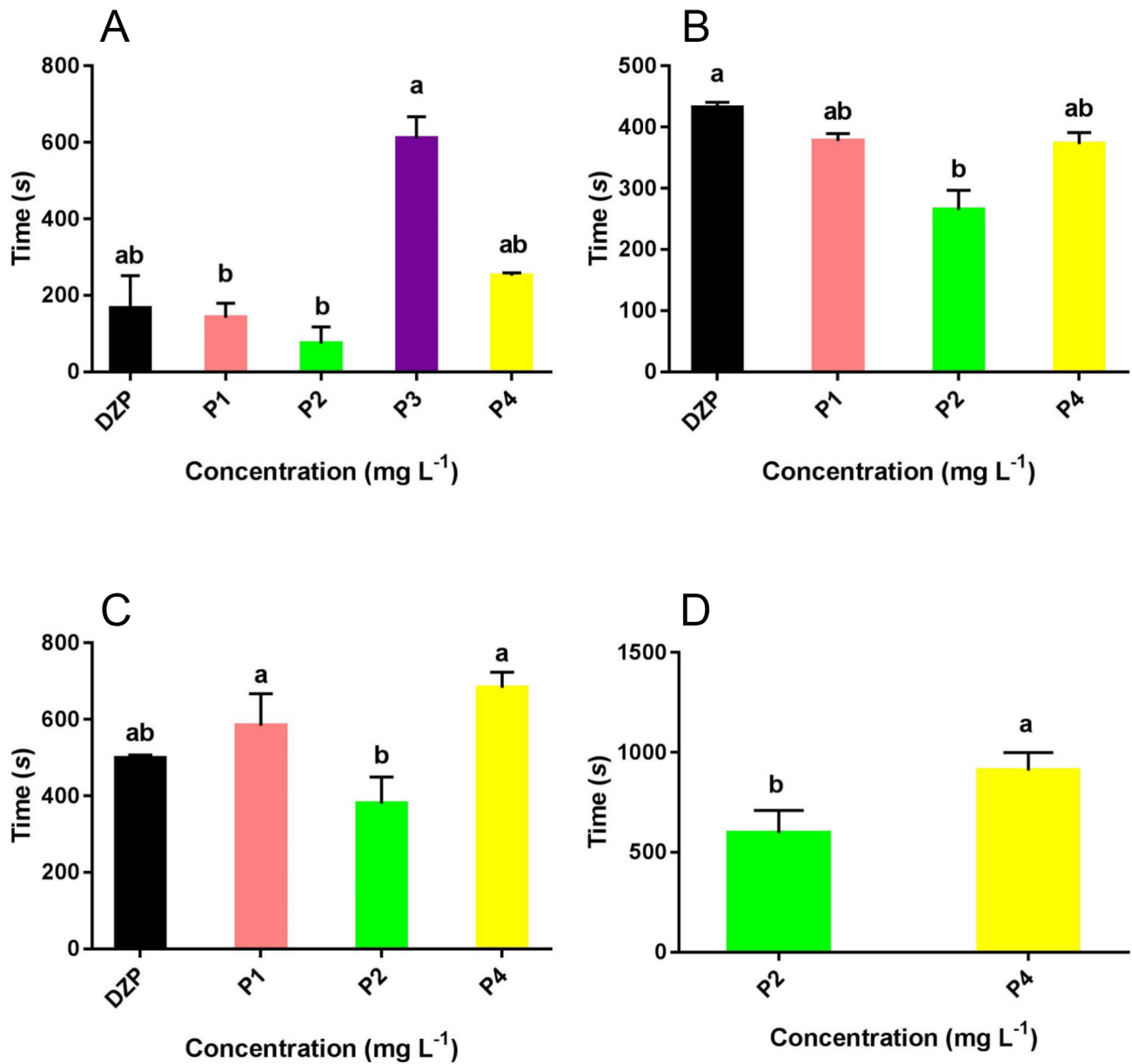


FIGURE 2 | Times to juveniles of *Rhamdia quelen* reach the stages S2 (A), S3a (B), S3b (C) and S4 (D) in response to diazepam (DZP) 150 μ M, DZP (150 μ M) associated to the sedative (P1) or anesthetic (P2) concentration of *Pilocarpus pennatifolius* essential oil from leaves (PPOL), and sedative (P3) and anesthetic (P4) concentrations of PPOL in the absence of DZP (n = 6). [P1 (DZP 150 μ M + PPOL 20 mg L⁻¹); P2 (DZP 150 μ M + PPOL 300 mg L⁻¹); P3 (PPOL 20 mg L⁻¹); P4 (PPOL 300 mg L⁻¹)].

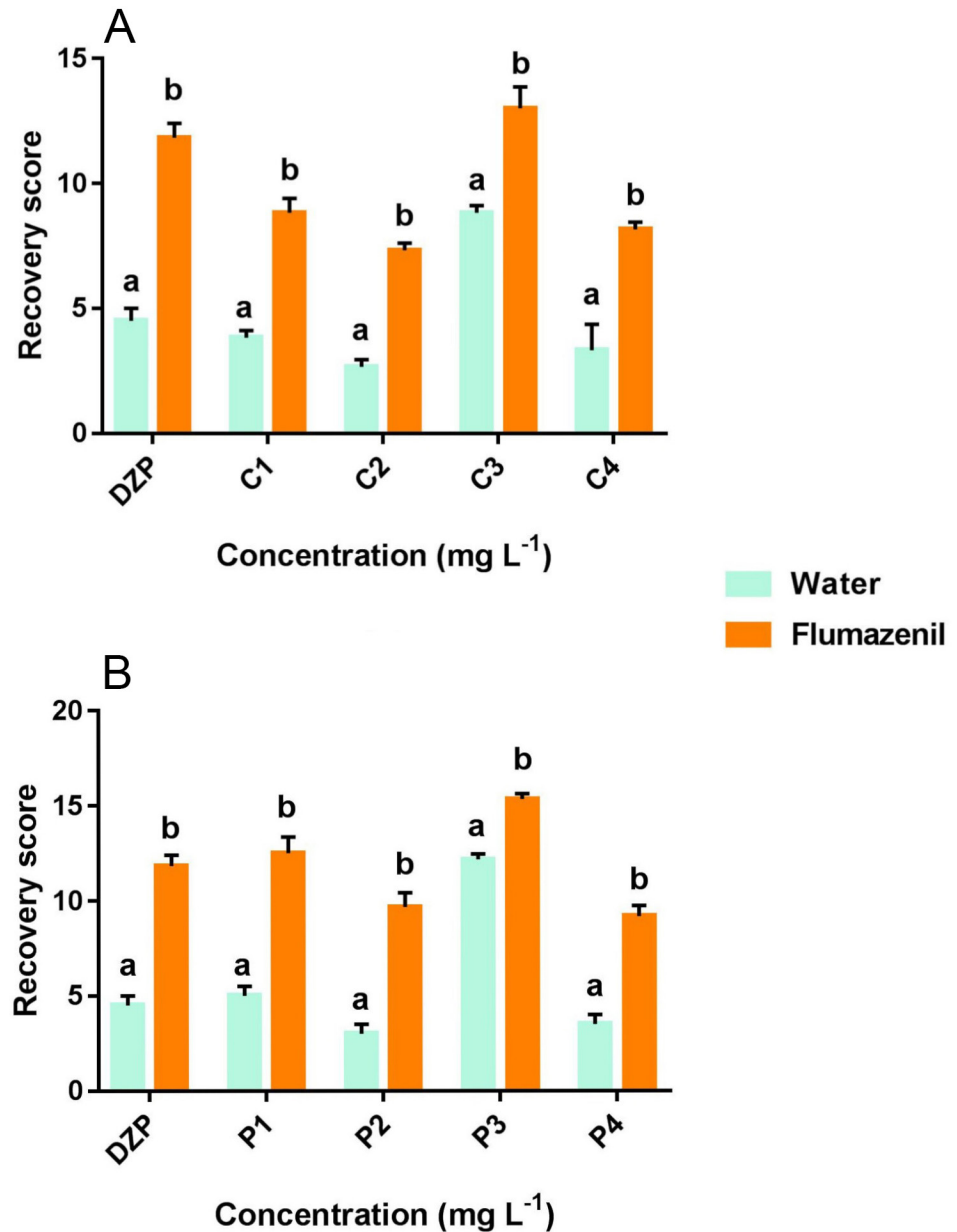


FIGURE 3 | Sum of the total recovery scores of fish ($n = 6$) exposed only to diazepam® (DZP 150 μM) or associated with essential oils (EOs) (C1; C2; P1; P2) and EOs in the absence of DZP at concentrations of 20 and 300 mg L^{-1} (C3; C4; P3; P4). The fish were exposed to these until they reached S4 or for a maximum time of 30 min and then placed in an aquarium containing only water ($n = 3$) or the benzodiazepine antagonist drug flumazenil® ($n = 3$). Refers to (A) CVOL – *Cordia verbenacea* essential oil from leaves [C1 (DZP 150 μM + CVOL 20 mg L^{-1}); C2 (DZP 150 μM + CVOL 300 mg L^{-1}); C3 (CVOL 20 mg L^{-1}); C4 (CVOL 300 mg L^{-1})] and (B) PPOL - *Pilocarpus pennatifolius* essential oil from leaves [P1 (DZP 150 μM + PPOL 20 mg L^{-1}); P2 (DZP 150 μM + PPOL 300 mg L^{-1}); P3 (PPOL 20 mg L^{-1}); P4 (PPOL 300 mg L^{-1})]. The scores were recorded at 1, 5, 10, 15 and 20 min, with the minimum score (0) being no sign of recovery and the maximum (3) fish swimming normally and showing reflexes after stimuli. Furthermore, according to Heldwein *et al.* (2012), the sum of the scores was added the value of 0.5 when the fish showed signs of agitation after the 20 min assessment. One-way ANOVA followed by Tukey's test. Different letters indicate statistical difference between water and flumazenil within each treatment.

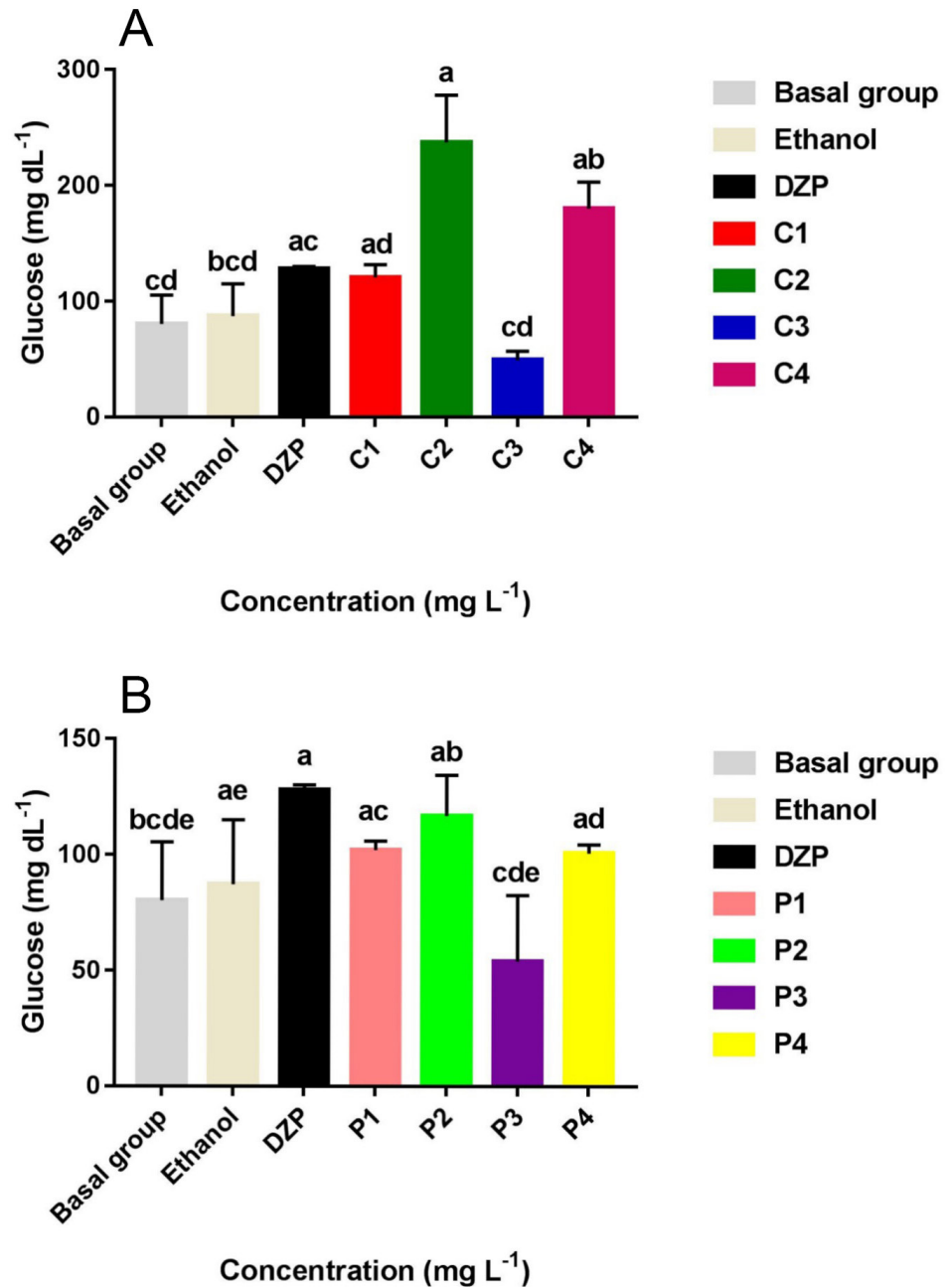


FIGURE 4 | Blood glucose levels in *Rhamdia quelen* juveniles exposed to diazepam® (DZP) 150 µM, ethanol (3000 µL L⁻¹), (A) CVOL - *Cordia verbenacea* essential oil from leaves [C1 (DZP 150 µM + CVOL 20 mg L⁻¹); C2 (DZP 150 µM + CVOL 300 mg L⁻¹); C3 (CVOL 20 mg L⁻¹); C4 (CVOL 300 mg L⁻¹)] and (B) PPOL - *Pilocarpus pennatifolius* essential oil from leaves [P1 (DZP 150 µM + PPOL 20 mg L⁻¹); P2 (DZP 150 µM + PPOL 300 mg L⁻¹); P3 (PPOL 20 mg L⁻¹); P4 (PPOL 300 mg L⁻¹)]. Measurements were also carried out on fish from the basal group (only exposed to water). Different letters indicate significant difference between treatments (n = 6).

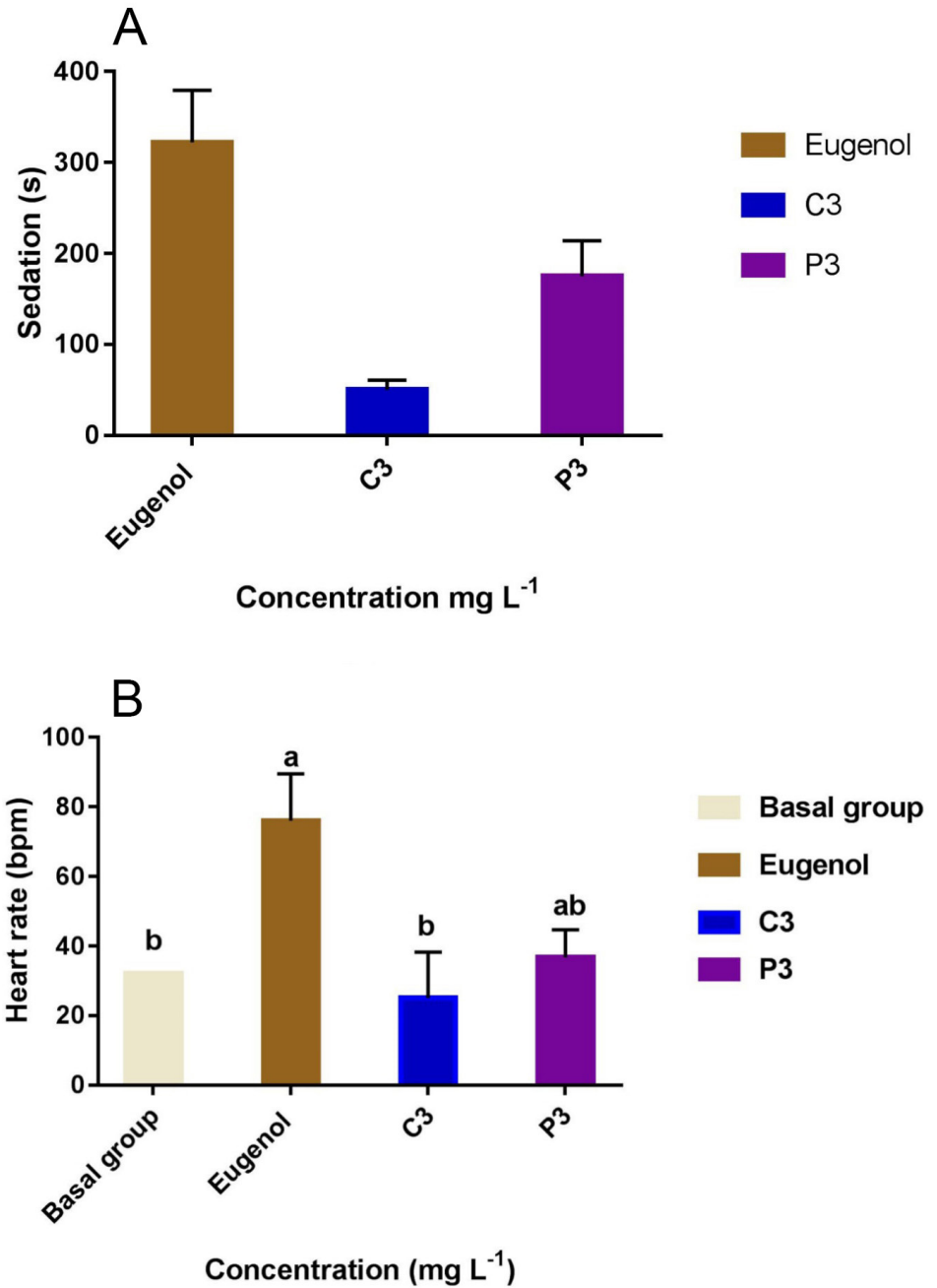


FIGURE 5 | Time to reach sedation (A) and (B) heart rate of *Rhamdia quelen* fish exposed to eugenol (50 mg L⁻¹) and to 20 mg L⁻¹ of the essential oils from the leaves of *Cordia verbenacea* (C3) and *Pilocarpus pennatifolius* (P3). The basal group does not appear in figure a because fish were only exposed to water. Different letters indicate significant difference between treatments (n = 8).

DISCUSSION

CVOL. CVOL has a proven anesthetic effect on native and exotic fish, such as juvenile silver catfish (Fortes *et al.*, 2024a) and Nile tilapia (Fortes *et al.*, 2024b). α -pinene (34.8%) was found in CVOL in a percentage similar (29.6%) to that described by Carvalho Jr. *et al.* (2004). This compound was previously observed in EOs from young (26.8%) and old (26.1%) leaves of *Nectandra megapotamica*, which induced sedation and anesthesia in *Centropomus parallelus*. Spathulenol, which was found in a low percentage in CVOL (2.8%), was also found in small amounts (1.2–2.1%) in the EO of *A. citriodora* and showed a direct relationship with time to induce deep anesthesia in silver catfish (Parodi *et al.*, 2020). The EOs from *N. megapotamica* leaves did not prevent the stress caused by anesthesia, according to the parameters evaluated (Tondolo *et al.*, 2013). In our study, the highest concentration of CVOL also apparently did not prevent stress, as there was an increase in glucose levels compared to the basal group.

The results indicate the possibility of a positive interaction between CVOL and DZP and participation of the BDZ site of GABA_A receptors in the effects observed with CVOL, highlighting the association of the sedative concentration (20 mg L⁻¹) with DZP (C1), which resulted in S4. Apparently, this is a strong indication of synergism or additive effect between the synthetic and natural agents, as C3 only resulted in S2 and DZP alone did not reach S4, signaling that the association possibly resulted in greater CNS depression. Another fact that could strengthen the suggestion of an additive effect or synergism between the drugs would be the 50% of fish that reached the S5 stage after exposure to C2, possibly due to deepening anesthesia (Garlet *et al.*, 2017). The anesthetic concentrations of CVOL with DZP apparently proved to be more effective in inducing the deeper anesthetic stages (S3b and S4), that is, faster than the isolated use of CVOL, being yet another possible evidence of additive or synergistic interaction between these compounds. Regarding the possible elucidation of the mechanism of action of CVOL, a significant statistical difference was found in the fish recovered in FLU compared to those that recovered in its absence (Heldwein *et al.*, 2012; Silva *et al.*, 2012), suggesting therefore the involvement of the benzodiazepine site of the GABAergic receptor complex in the effects observed for CVOL.

The data found in the literature reinforces these results, as some studies indicate that α -pinene (Aoshima, Hamamoto, 1999) is a positive modulator of GABA_A receptors. Furthermore, CVOL presents spathulenol at 2.8%, a constituent that has already been indicated with possible GABAergic involvement in its central depressant effects in rodents and fish (Oliveira Júnior *et al.*, 2018; Garlet *et al.*, 2019a). However, other possible mechanisms of action and interactions cannot be ruled out, as CVOL has other compounds that have not been identified. A factor to highlight in these EOs is the recovery times, as in studies with tilapia (Fortes *et al.*, 2024b) and silver catfish (Fortes *et al.*, 2024a) they were quite prolonged, that is, it could be an indication of high potency. This is because, when analyzing synthetic drugs, the most potent are those that recover later, for example, fish in an immersion bath with propofol recover later than those in an immersion bath with benzocaine (Gonçalves, Giaquinto, 2020). This reflects the high potency of propofol compared to benzocaine, therefore, the choice of inducing agent will depend on the need for the duration of central depression (Gonçalves, Giaquinto, 2020). The association of EOs with synthetic pharmacological agents, such as DZP,

has also been observed in other studies as a strategy to improve anesthetic efficacy (Silva *et al.*, 2012; Heldwein *et al.*, 2014; dos Santos *et al.*, 2017). Furthermore, the faster and more profound effect presented in this study when associating DZP with CVOL demonstrates this trend and can be explained by the interaction of DZP with the benzodiazepine site in the CNS, which apparently facilitates the sedative and anesthetic action of EOs, as observed in this study. The interaction between the compounds seems to be essential for obtaining faster and more effective responses.

The higher blood glucose levels in fish from the C2 group suggests that C2 caused stress to the juveniles. A different result was observed in fish exposed to P2, a sample that corresponds to the association of DZP + PPOL, which did not increase the glucose levels of juveniles compared to the basal group. Thus, the results indicate that CVOL associated with DZP cannot be used for deep anesthesia in juvenile silver catfish, as it apparently leads to strong central depression, causes stress and death when anesthesia is not reversed with a BZP antagonist, such as flumazenil. Therefore, when longer procedures need to be performed for example, transport or something that requires longer-lasting sedation for the fishing industry, the use of CVOL could be suggested. Furthermore, the sedative concentration in silver catfish juveniles did not increase blood glucose levels compared to the basal group and when evaluated in fish of larger size and weight, it caused good deep sedation and did not change heart rate compared to the basal group, unlike what was observed for eugenol. In this sense, the sedative concentration of 20 mg L⁻¹ can be used to transport juvenile silver catfish and also larger fish. However, more stress parameters must be evaluated, such as plasma cortisol and others.

PPOL. Sedative and/or anesthetic activities have not yet been described for 2-Undecanone (57.2%) and 2-Tridecanone (28.3%), the main compounds found in PPOL. However, germacrene D (10.4%) was identified in EOs from young (9.1%) and old (9.2%) leaves of *N. megapotamica* (Tondolo *et al.*, 2013) and also in the EO of *Hyptis mutabilis* (Silva *et al.*, 2013), which presented sedative and anesthetic effects, respectively, on *C. parallelus* and silver catfish. Previous studies that analyzed the chemical composition of PPOL have reported the predominance of Tridecane (56.8%), Pentadecane (25.5%) and Spathulenol (2.6%) (Santos *et al.*, 2004). The majoritarian composition of PPOL, when considered the biosynthetic origin, is quite similar to that found in our work. However, in PPOL, the simple hydrocarbon derivatives underwent oxygenation producing ketones, which were not described by Santos *et al.* (2004). Differences in the chemical composition of essential oils from the same species can be explained by genetic differences expressed in different populations, which justify the occurrence of distinct chemotypes, as well as geographical and environmental influences, plant development conditions and processing methods, among other factors (Pant *et al.*, 2021; Etri, Pluhár, 2024). Spathulenol was found in PPOL at 0.6%. This is also a compound indicated for contributing to the anesthetic activities of the EOs of *Aloysia gratissima* (Benovit *et al.*, 2015) and *N. grandiflora*, which have anesthetic and sedative activity in silver catfish (Garlet *et al.*, 2019a). However, a possible contribution of sedative or anesthetic activity of 2-Undecanone and 2-Tridecanone cannot be ruled out. Thus, the anesthetic activity of these compounds could be investigated, as promising results can be found, considering that no changes were found in glucose levels and no difference in heart rate of fish exposed to PPOL compared to the basal

group could be detected. No adverse effects, side effects, or abnormal clinical and behavioral signs were found with the use of PPOL in silver catfish or tilapia (Fortes *et al.*, 2024b). In this sense, PPOL could be recommended in the future in procedures such as medication and vaccine applications that require sedation and/or anesthesia for faster recovery, as this is within the indications of some researchers regarding induction and recovery time (Keene *et al.*, 1998; Roubach *et al.*, 2001; Obirikorang *et al.*, 2020). In view of the elucidation of the possible mechanism of action of PPOL and possible positive interaction with DZP, it was seen that P3 differed from P1 in the evaluation of stratification in S2, as P1 led to this stage in a shorter time. Furthermore, in S3b P4 differed from P2, that is, when associated with DZP, the time to reach S3b was shorter. Furthermore, to achieve deep anesthesia there was a statistically significant difference between P2 and P4, as S4 was reached more quickly when combined with DZP. In this sense, we offer evidence that suggests a positive interaction of PPOL concentrations with DZP, which was demonstrated in decreasing time to reach the CNS depression stages. Another fact to be highlighted regarding the modulation of the anesthetic effect of PPOL associated with DZP is the evidence that the fish were taken to deeper stages.

The possible mechanism of action of PPOL in view of the evidence from this study seems to involve the benzodiazepine site of the GABA_A receptor complex, however no description of the action was found for the two major constituents of PPOL, 2-Undecanone and 2-Tridecanone. A study with the EO of *Croton conduplicatus* leaves, which presents spathulenol (15.47%) as main compound, suggests the involvement of GABA_A receptors in mice as a possible mechanism of action (Oliveira Júnior *et al.*, 2018). Another study, in which spathulenol was present in 3.48% of the EO, sedated silver catfish and it was suggested that its effect was probably the result of GABA_A-mediated action (Garlet *et al.*, 2019a). In this context, it may be that this compound present in PPOL has contributed to the possible involvement of the GABAergic receptor, observed in silver catfish juveniles. These findings offered by our study are in agreement with several pharmacological reports of possible interaction of sesquiterpenes and monoterpenes in GABA/BZP mediation (Granger *et al.*, 2005; Rivera *et al.*, 2014; Sousa *et al.*, 2015; Milanos *et al.*, 2017). More specifically in silver catfish, it was also suggested that sesquiterpenes present in the EO of *N. grandiflora* modulated GABAergic activity (Garlet *et al.*, 2019a). However, other mechanisms of action cannot be ruled out since there is a lack of studies with the two major compounds of PPOL and the participation of spathulenol in possible interactions with muscarinic receptors (M2, M3 and M4) and opioids (Delta and Mu) (Oliveira-Júnior *et al.*, 2018), for example. Thus, given the complex chemical composition of EOs, the mechanism of action may result from the interaction of different components with more than one system, depending on the chemical composition. Another important point of our study is the recovery time, as the results showed that fish recovered in FLU more quickly and efficiently compared to those that recovered in water. The data reinforce the role of benzodiazepine antagonists in reversing sedative and anesthetic effects. Furthermore, the fish recovered satisfactorily, with a return to normal feeding after 24 h, indicating that treatments with EOs did not cause prolonged adverse effects. This is yet another positive indication for the use of these compounds in aquaculture. PPOL showed promising effects in the analysis of glucose levels in all its concentrations, compared to the basal group and in the *hr* evaluation experiment used in this study, contrary to what was observed for eugenol.

The methodology of evaluating *hr* with Doppler ultrasound used in our work was recently used to demonstrate that anesthesia with MS-222 (300 mg L⁻¹) and the EO of *Protium heptaphyllum* (PHEO) (600 mg L⁻¹) increased *hr* in silver catfish (da Silva *et al.*, 2024). While fish anesthetized with CVOL and PPOL did not showed change in *hr* compared to the control, eugenol increased it. In this sense, eugenol may cause a transient sympathetic activation that significantly elevates *hr* as seen with PHEO. This may be due to the ability of eugenol and PHEO to interact with adrenergic receptors and induce the release of catecholamines such as adrenaline and noradrenaline (Joyce *et al.*, 2023). Therefore, the cardiovascular effects observed in the study are extremely relevant to the safety of using these EOs. This is because the increase in *hr* with the use of eugenol was observed, however in the groups exposed to CVOL (C3) and PPOL (P3) no significant changes were observed. Thus, these results suggest that eugenol may have a stimulating effect on the cardiovascular system, while CVOL and PPOL do not induce significant changes in *hr*. Therefore, the use of these EOs can be considered an advantage in terms of safety for the species under study. Ultimately, this device was satisfactory for determining *hr* and we also suggest its use for other cardiac assessments in fish, such as myocardial contractions in catfish sedated and/or anesthetized with EOs from Brazilian biodiversity and other countries.

The results of the study demonstrate that concentrations of CVOL and PPOL are effective in inducing sedation and anesthesia, but when associated with DZP, they may be more effective in inducing sedation and anesthesia in *Rhamdia quelen*. The positive interaction between these compounds may allow a faster and more effective induction of deep anesthetic stages, while recovery was accelerated with the use of flumazenil. In this sense, the results suggest the involvement of the benzodiazepine site of the GABAergic receptor complex for both CVOL and PPOL in silver catfish sedation and anesthesia, but further studies regarding the activation of CNS receptors for these EOs need to be carried out. The anesthetic concentration of CVOL increased blood glucose levels compared to the basal group and after deep anesthesia apparently decreased feed consumption, indicating that it did not prevent stress in the fish. The sedative concentration did not change glucose levels and also did not increase heart rate. Apparently CVOL has potential to be used in sedative concentration (20 mg L⁻¹). In the case of PPOL, the results were promising for the development of a sedative/anesthetic for juvenile silver catfish and larger sizes (around 35 cm) for sedation 20 mg L⁻¹ and for deep anesthesia 300 mg L⁻¹, as blood glucose levels and *hr* did not increase compared to the basal group. Although the effects on blood glucose levels and heart rate require further investigation, the findings suggest that these EOs have potential for use in aquaculture as alternatives to conventional anesthetics. Essentially, as they offer a less invasive and safer approach, no adverse effects, mortality or altered clinical and behavioral signs were found for the concentrations suggested above.

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AUTHORS' CONTRIBUTION

Carlos Herminio Magalhães Fortes: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing-original draft, Writing-review and editing.

Bernardo Baldisserotto: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing-review and editing.

Frederico Dimas Fleig: Resources, Writing-review and editing.

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ETHICAL STATEMENT

The methodology was approved by the UFSM Ethics Committee under nº 9303280623 and the project was registered in SISGEN under nº AE4FE76.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

COMPETING INTERESTS

The authors declare no competing interests.

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