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Abstract

The objective of this study was to identify the possible involvement of the GABAergic system in the anesthetic effect of *Lippia alba* essential oil (EO). We propose a new animal model using silver catfish (*Rhamdia quelen*) exposed to an anesthetic bath to study the mechanism of action of EO. To observe the induction and potentiation of the anesthetic effect of EO, juvenile silver catfish (9.30 ± 1.85 g; 10.15 ± 0.95 cm; N = 6) were exposed to various concentrations of *L. alba* EO in the presence or absence of diazepam [an agonist of high-affinity binding sites for benzodiazepinic (BDZ) sites coupled to the $GABA_A$ receptor complex]. In another experiment, fish (N = 6) were initially anesthetized with the EO and then transferred to an anesthetic-free aquarium containing flumazenil (a selective antagonist of binding sites for BDZ coupled to the $GABA_A$ receptor complex) or water to assess recovery time from the anesthesia. In this case, flumazenil was used to observe the involvement of the GABA-BDZ receptor in the EO mechanism of action. The results showed that diazepam potentiates the anesthetic effect of EO at all concentrations tested. Fish exposed to diazepam and EO showed faster recovery from anesthesia when flumazenil was added to the recovery bath (12.0 ± 0.3 and 7.2 ± 0.7, respectively) than those exposed to water (9.2 ± 0.2 and 3.5 ± 0.3, respectively). In conclusion, the results demonstrated the involvement of the GABAergic system in the anesthetic effect of *L. alba* EO on silver catfish.

Key words: False-melissa; Silver catfish; Benzodiazepines; Flumazenil; GABA

Introduction

Various substances or combinations of substances, such as barbiturates, quinaldine, tricaine methanesulfonate (MS 222), metomidate, etomidate, and benzocaine, have been used to induce anesthesia in different fish species to minimize stress in these animals (1-4). However, in recent years, natural products such as clove oil and isoeugenol (AQUI-S), have demonstrated similar or superior effectiveness with less toxicity to fish compared to anesthetic drugs (5,6).

Lippia alba (Mill.) N.E. Brown, known as "false-melissa" in Brazil, is an aromatic shrub whose great chemical variability also leads to variations in its pharmacological activity. In Brazil, three main chemotypes are reported and classified according to their major constituent as citral, carvone and linalool (7).

This plant has been commonly used for its sedative properties, which have been demonstrated in some rodent studies (7,8). Recently, the anesthetic effect of *L. alba* essential oil (EO) was demonstrated on silver catfish (*Rhamdia quelen*). The EO of *L. alba* is considered to be a novel natural sedative and anesthetic agent that can be potentially used in aquaculture practices due to its ability to reduce stress in fish with a consequent reduction of economic losses in fish culture (9).

Most anesthetics exert their effects by regulating the gamma-aminobutyric acid (GABA) receptor complex, as

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GABA is the main inhibitory neurotransmitter in the central nervous system (10). Synaptic GABA release activates $GABA_A$ and $GABA_C$ ionotropic receptors as well as the metabotropic receptor $GABA_B$ (11,12). $GABA_A$ receptors have been implicated in the majority of GABAergic signaling in mammals. High-affinity binding sites for benzodiazepines (BDZ) are known as "central BDZ receptors", and these compounds exert their pharmacological action by allosteric modulation in these regions. The activation of these sites enhances GABA-induced chloride ion flux, resulting in hyperpolarization and central depression. Expression of the GABAergic system seems to be conserved among vertebrates, from fishes to mammals (13). This system has been detected in certain parts of the zebrafish brain (13,14) and could be involved in their response to anesthetics. The mechanism of action of *L. alba* EO has not yet been elucidated. In order to determine the mechanism of the anesthetic effect of EO, we propose a new animal model using silver catfish to examine the involvement of the GABAergic mechanism in fish exposed to an anesthetic bath.

Material and Methods

Plant material

L. alba was grown at the CESNORS/UFSM Campus, Frederico Westphalen, southern Brazil (geographically situated at 27°23'26" South, 53°25'43" West and 461 m above sea level). The species was identified by Gilberto Dolejal Zanetti, Department of Industrial Pharmacy, UFSM, and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Department of Biology, UFSM.

EO extraction and analysis

EO was obtained from fresh *L. alba* leaves using the hydrodistillation process with a Clevenger type apparatus (15) for 2 h. The yield was calculated as w/w (%). The EO was stored at -20°C until composition analysis and testing. For comparison purposes, the composition of the EO used in this study (EO1) and the corresponding data for the EO used by Cunha et al. (9) (EO2) are presented in Table 1. The compositions of both EOs were analyzed by GC-MS.

GC-MS TIC analysis was performed using an Agilent-6890 gas chromatograph coupled to an Agilent 5973 mass selective detector, under the following conditions: HP-5MS column (5% phenyl-95% methylsiloxane, 30 m x 0.25 mm x 0.25 µm); EI-MS: 70 eV; operating conditions: split inlet, 1:100; temperature program, 40-260°C; 40°C for 4 min; ramp rate, 4°C/min; carrier gas, He; flow rate, 1 mL/min; injector and detector temperature, 220°C; interface temperature, 250°C.

The constituents of the EOs were identified by comparison of the mass spectra with a mass spectral library (16) and by comparison of the Kovats retention index with literature data (17).

Animals

Juvenile silver catfish (9.30 ± 1.85 g; 10.15 ± 0.95 cm) were housed in continuously aerated 250-L tanks (18.46 ± 0.51°C; pH 7.47 ± 0.073; total ammonia levels, 0.0883 mg/L; dissolved oxygen levels, 8.84 ± 0.37 mg/L). A semi-static system was used, and 50% of the water volume was changed daily. The fish received a commercial diet (Vicente Alimentos S.A., Brazil) with 3.5% Ca^{2+}, 28.0% crude protein and 3500 kcal/kg digestible energy, as stated by the manufacturer. Juveniles were fed once a day and were fasted for a period of 24 h prior to the experiments. The water parameters were measured before the experiments. Dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512; YSI Inc., USA). The pH was verified with a DMPH-2 pH meter (Digimed, Brazil) and nesslerization was used to verify total ammonia levels according to the method of Greenberg et al. (18).

The experiments were approved by the Ethics and Animal Welfare Committee of Universidade Federal de Santa Maria (protocol No. 23081.014553/2009-39).

Standardization of the test conditions

The depressor effect of anesthetics in fish was evaluated by the method of Schoettger and Julin (19). The method involves six stages, in which the following parameters were observed: light and deep sedation (stages 1 and 2, respectively), partial and total loss of equilibrium (stages 3a and b, respectively), deep anesthesia (stage 4) and medullar collapse (stage 5).

Prior to the potentiation test, diazepam concentration (BDZ) (DEG, Brazil) was standardized. Fish were transferred to 1-L aquaria containing 25, 100, and 150 µM BDZ, previously solubilized in 0.033% Tween 80. To evaluate the time required to reach the different stages of anesthesia induction, three juveniles were used for each concentration tested, and each juvenile was used only once, according to the method of Schoettger and Julin (19). The maximum time of observation was 30 min. After induction, animals were transferred to an anesthetic-free aquarium to assess the recovery time.

For tests of the reversal of anesthetic effects, the flumazenil (Flumazil®, Cristália, Brazil) concentration in the recovery bath was also standardized and used to observe the involvement of the GABA-BDZ receptor in the mechanism of action of EO. Fish (N = 6) were subjected to sedation induction with 150 µM BDZ in 1-L aquaria. After induction, the animals were divided into two groups: one group was transferred to an anesthetic-free aquarium with water while the second group was placed in an aquarium containing flumazenil. In both groups, fish behavior was scored after 1, 5, 10, 15, and 20 min, as described in Table 2. After 20 min, the sum of scores for each fish was calculated. When agitation was verified in a given fish, we added 0.5 to its score. Each observation included an external stimulus while observing the fish; undisturbed animals tend to remain

Table 1. Chemical constituents of *Lippia alba (*Mill.) N.E. Brown essential oil used in the present study (EO1) and by Cunha et al. (9) (EO2).

Components	Relative %		Retention time (min)		IK calculated		IK literature
	EO1	EO2	EO1	EO2	EO1	EO2	
α-thujone	-	0.04	-	10.49	-	925	930[a]
α-pinene	0.13	0.23	10.10	10.75	911	931	939[a]
camphene	0.20	0.50	10.67	11.42	927	947	954[a]
α-phellandrene	1.32	-	11.76	-	954	-	1003[a]
sabinene	0.22	1.90	11.83	12.42	956	972	975[a]
1-octen-3-ol	0.10	-	12.15	-	964	-	979[a]
myrcene	0.84	1.37	12.58	13.17	974	990	991[a]
limonene	0.48	1.11	14.06	14.75	1007	1029	1029[a]
1,8-cineole	9.11	8.59	14.18	14.95	1011	1034	1031[a]
β-ocimene, *Z*	0.09	0.13	14.52	15.09	1021	1038	1037[a]
β-ocimene, *E*	0.67	0.62	14.93	15.49	1032	1048	1050[a]
γ-terpinene	0.03	0.04	15.30	15.94	1041	1059	1060[a]
sabinene hydrate, *Z*	0.14	-	15.66	-	1051	-	1070[a]
linalool oxide, *E*	-	0.47	-	16.51	-	1073	1073[a]
isoterpinolene	-	0.48	-	16.97	-	1085	1088[a]
linalool oxide, *Z*	0.05	0.16	15.92	17.15	1057	1089	1087[a]
terpinolene	0.22	-	16.49	-	1071	-	1089[a]
sabinene hydrate, *E*	0.02	-	16.94	-	1082	-	1098[a]
linalool	59.66	37.47	17.33	18.00	1091	1111	1097[a] 1094[b]
3-hexenyl isobutanoate, *Z*	-	0.03	-	19.20	-	1143	1147[a]
hotrienol	0.10	-	17.38	-	1092	-	1104[b]
1,3,8-*p*-menthatriene	0.07	-	17.91	-	1106	-	1110[a]
6-camphenol	0.04	-	18.08	-	1111	-	1114[a]
camphor	3.15	6.87	18.76	19.53	1130	1152	1146[a] 1144[b]
chrysanthenol, *E*	0.03	-	19.23	-	1143	-	1164[a]
bicyclo[2.2.1] heptan-3-one,6,6-dimethyl, 2-methylene	0.29	-	19.46	-	1149	-	1149[b]
pinocarvone	-	0.66	-	20.03	-	1165	1165[a]
borneol	0.37	1.06	19.58	20.45	1153	1176	1169[a]
p-mentha-1,5-dien-8-ol	0.65	-	19.65	-	1155	-	1170[a]
neoiso-isopulegol	0.09	-	19.96	-	1163	-	1171[a]
terpinen-4-ol	0.07	-	20.03	-	1165	-	1177[a]
α-terpineol	0.58	0.88	20.56	21.31	1178	1199	1189[a]
myrtenal	0.11	0.09	20.76	21.25	1183	1198	1196[a]
2,6-dimethyl-3,5,7-octatrien-2-ol, *Z*, *Z*	0.64	-	21.01	-	1190	-	1090[b]
2,6-dimethyl-3,5,7-octatrien-2-ol, *E*, *E*	1.65	-	21.31	-	1197	-	1134[b]
verbenone	-	0.36	-	21.74	-	1211	1205[a]
trans-carveol	-	2.00	-	21.88	-	1215	1217[a]
citronellol	0.02	-	22.04	-	1219	-	1226[a]
neral (citral b)	0.15	-	22.48	-	1232	-	1238[a]
geranial (citral a)	0.14	-	23.57	-	1263	-	1267[a]
isobornyl acetate	0.07	0.16	24.11	24.36	1278	1285	1286[a]

Continued on next page

Table 1 continued.

Components	Relative %		Retention time (min)		IK calculated		IK literature
	EO1	EO2	EO1	EO2	EO1	EO2	
myrtenyl acetate	-	0.03	-	25.70	-	1325	1327[a]
δ-elemene	0.03	0.03	25.93	26.03	1332	1335	1338[a]
exo-2-hydroxycineolacetate	0.11	-	26.12	-	1338	-	1354[b]
α-cubebene	0.01	0.02	26.36	26.42	1345	1346	1351[a]
cyclosativene	-	0.04	-	27.15	-	1368	1371[a]
α-copaene	0.19	0.38	27.26	27.38	1373	1375	1377[a]
β-bourbonene	0.17	0.12	27.58	27.64	1382	1383	1388[a]
β-cubebene	-	0.19	-	27.78	-	1387	1388[a]
β-elemene	1.32	0.69	27.84	27.84	1390	1389	1391[a]
α-gurjenene	0.023	0.07	28.43	28.38	1407	1406	1410[a]
β-caryophyllene	2.84	4.09	28.77	28.84	1419	1420	1419[a]
β-copaene	0.11	0.15	29.07	29.12	1429	1429	1432[a]
γ-elemene	0.91	-	29.22	-	1434	-	1437[a]
α-humulene	0.364	0.89	29.88	29.95	1455	1456	1455[a]
β-farnesene, *E*	0.21	-	29.97	-	1458	-	1458[b]
allo-aromadendrene	0.17	0.28	30.12	30.07	1463	1460	1460[a] 1459[b]
γ-muurolene	-	0.16	-	30.58	-	1476	1480[a]
α-amorphene	0.11	-	30.66	-	1479	-	1485[a] 1480[b]
germacrene D	3.78	5.40	30.82	30.80	1484	1483	1485[a] 1486[b]
4-epi-cubebol	0.09	-	31.23	-	1497	-	1494[a]
bicyclogermacrene	0.14	0.27	31.28	31.19	1499	1495	1500[a]
α-muurolene	0.23	0.58	31.40	31.28	1502	1498	1500[a]
germacrene A	-	0.88	-	31.58	-	1508	1509[a]
10-epi-cubebol	0.42	-	31.89	-	1520	-	1535[a]
γ-cadinene	0.36	0.06	32.13	31.73	1529	1513	1514[a]
δ-cadinene	-	1.16	-	31.88	-	1518	1523[a]
nerolidol, *Z*	0.18	-	32.60	-	1545	-	1533[a]
germacrene B	-	3.65	-	33.14	-	1561	1561[a]
nerolidol, *E*	-	0.58	-	33.23	-	1564	1563[a]
germacrene D-4-ol	1.05	2.56	33.79	33.71	1585	1580	1576[a]
caryophyllene oxide	0.62	0.91	34.03	33.84	1593	1584	1583[a]
viridiflorol	0.06	-	34.65	-	1616	-	1593[a]
humulene epoxide II	-	0.15	-	34.64	-	1612	1608[a]
1,10-di-epi-cubenol	-	0.12	-	34.79	-	1617	1619[a]
τ-cadinol	-	0.17	-	35.56	-	1645	1640[a]
τ-muurolol	0.21	0.53	35.76	35.62	1656	1647	1642[a]
α-muurolol	-	0.18	-	35.70	-	1650	1646[a]
14-hydroxy-9-epi-caryophyllene, *E*	0.17	-	35.99	-	1664	-	1670[a]
α-cadinol	0.25	0.62	36.14	35.96	1670	1659	1654[a]
14-hydroxy-α-muurolene	0.26	-	39.11	-	1780	-	1780[a]
Σ (%) of identified compounds	95.92	90.18					

IK = Kovats retention index. [a]Adams (17). [b]NIST Databank 2002 (16).

stationary. This stimulation consisted of successive hits with a glass rod on the bottom of the aquarium.

Experiment 1: Potentiation with BDZ

Various concentrations (50, 100, and 300 µL/L) of *L. alba* EO (previously diluted 1:10 in ethanol) were added along with BDZ (150 µM) to the 1-L aquaria. Six fish were tested individually for each concentration. Evaluation of BDZ with ethanol (EO vehicle), EO and both vehicles alone (Tween 80 and ethanol at the same proportions used) was also performed. The stages of anesthesia induction and recovery time were evaluated according to the protocol described in the standardization of the test conditions.

Experiment 2: Reversal of anesthetic effects

The same protocol described in the standardization of the test conditions was repeated with *L. alba* EO at 300 µL/L. After induction, fish (N = 6) were divided into two groups: one group was transferred to an anesthetic-free aquarium with water, and the second group was placed in an aquarium containing 5 µM flumazenil. The results were scored (Table 2) after 1, 5, 10, 15, and 20 min and are reported as the sum of scores.

Statistical analyses

All data were subjected to a Levene test to determine the homogeneity of variances. One-way ANOVA followed by the Tukey test or Kruskal-Wallis and Mann-Whitney tests were used in the potentiation test when appropriate (SPSS version 17.0). Results of the study of reversal of the anesthetic effects were compared by two-way ANOVA and the Tukey test with Sigma Plot (version 11.0). Significance was set at the level of 95% ($P < 0.05$). Data are reported as means ± SEM.

Table 2. Scores of recovery from anesthesia in fish.

Scores	Behavior
0	No sign of recovery
0.5	Reaction only after a caudal peduncle stimulus
1	First sign of recovery but without posture
1.5	Stopped after erratic swimming
2	Normal swimming, but without reflex after an external stimulus
2.5	Stopped after normal swimming, but without reflex after an external stimulus
3	Normal swimming with reflex after an external stimulus

Results

EO composition

The EO yield (w/w) was 0.35% based on its mass in relation to the mass of plant material. The chemical composition of the EO used in this study (EO1) is similar to the corresponding data of the EO used by Cunha et al. (9) (EO2). However, EO1 contained 62 identified compounds, whereas EO2 contained 48 compounds. Monoterpene compounds represent 81.53% of EO1 and 65.22% of EO2, and sesquiterpenes represent 14.29 and 24.93% of EO1 and EO2, respectively. The major components of EO1 and EO2 were linalool (59.66 and 37.47%, respectively), 1,8-cineole (9.11 and 8.59%), camphor (3.15 and 6.87%), germacrene D (3.78 and 5.40%), and β-cariophyllene (2.84 and 4.09%; Table 1).

Standardization of the test conditions

Fish exposed to 25 µM BDZ reached deep sedation (stage 2), whereas those exposed to 100 and 150 µM presented total loss of equilibrium (stage 3a). Silver catfish exposed to the concentrations of diazepam used in this study did not reach stage 4 of anesthesia. The concentration of 150 µM was chosen for the subsequent tests because fish reached stage 3a within a relatively short period of time (about 9 min) and showed a longer recovery time (about 18 min) compared to those exposed to 100 µM BDZ (induction time approximately 22 min; recovery time approximately 5 min). A longer recovery time was required to better determine the efficacy of the antagonist in the reversal of the anesthetic effects.

The flumazenil concentration was standardized by testing 0.05, 1.0, and 5.0 µM of this GABA receptor antagonist. Scores of recovery from anesthesia in fish exposed to 1 µM (10.2 ± 0.6) and 5 µM flumazenil (9.3 ± 0.6) were significantly different from those of control animals (8.2 ± 0.4 and 7.8 ± 0.4, respectively) with $P < 0.05$. Fish that recovered in water with 0.05 µM flumazenil showed scores (8.5 ± 0.3) that were not significantly different from animals that recovered in water alone (7.8 ± 0.3). In this test, higher scores indicate faster recovery from the anesthesia.

The application of ethanol alone did not produce an anesthetic effect.

Experiment 1: Potentiation with BDZ

The combination of BDZ+EO of *L. alba* significantly reduced the time required to reach anesthesia compared to the EO alone. BDZ potentiated deep sedation (stage 2) and total loss of equilibrium (stage 3a) of the animals exposed to *L. alba* EO, except in the case of fish exposed to 100 µL/L regarding stage 2 (Figure 1A and B). Given alone, 50 µL/L EO only led to stage 3a anesthesia, but, in combination with BDZ, it led to stage 4 anesthesia. The highest EO concentrations evaluated (100 and 300 µL/L) also led to stage 4, and combined treatment with BDZ decreased the time required to reach this stage (Figure 1C). The time of recovery from *L. alba* EO anesthesia was significantly

longer in fish exposed to 300 µL/L than to the lower EO concentrations, and the combination of BDZ+EO of *L. alba* did not change the recovery time (Figure 1D).

Experiment 2: Reversal of the anesthetic effects

Fish anesthetized with *L. alba* EO (300 µL/L) or exposed to BDZ presented significantly higher recovery score sums when recovering in water with flumazenil than in water alone ($P < 0.05$; Figure 2). Therefore, recovery was faster in water containing flumazenil.

Discussion

The EO used in this study, as well as that used by Cunha et al. (9), was obtained from *L. alba* chemotype linalool, which was previously described by Frighetto et al. (20) and Duarte et al. (21). When comparing the composition of the EO used in this study to that used by Cunha et al. (9), we observed similar chemical compositions. Although there were discrepancies between our results and previous results regarding the percentages of the major EO components, the effects observed for the natural anesthetic mixture without BDZ were as expected, and we demonstrated that the EO used in our assays can reproduce the effect observed by Cunha et al. (9). However, the time to achieve stage 4 was longer in the present study than in the study by Cunha et al. (9). This disparity can be explained by the different percentage of some compounds, including the major compound (linalool).

GABA complex ligands include agonists, antagonists and modulators. Typically, some behavioral effects that result from positive allosteric modulators of $GABA_A$ receptors are anxiolysis, cessation of convulsions, sedation, and general anesthesia (22-24). All of these activities have been described for *L. alba* EO (7-9,25,26). Additionally, Vale et al. (7) verified that EOs of *L. alba* belonging to limonene and citral chemotypes have similar pharmacological profiles to those of BDZ, indicating that they might interact with the binding sites for benzodiazepines located adjacent to the $GABA_A$ receptor complex (11,12).

This potential GABAergic involvement was evaluated by the potentiation of the anesthetic effect of *L. alba* EO with BDZ. The best result observed in the present study was produced by the induction of anesthesia with the lowest concentration of EO tested. This clearly indicates the synergism of the compounds and the possibility for the combined use of these products to reduce the concentration of both in fish anesthesia. Additionally, the results indicate that the EO of *L. alba* has some effect on the GABA-receptor complex. This finding was confirmed by the faster recovery period observed with flumazenil treatment after the EO bath. In the animal model developed in the present study, the recovery scores recorded after 20 min of observation were summed and the values were converted to percentages. In these

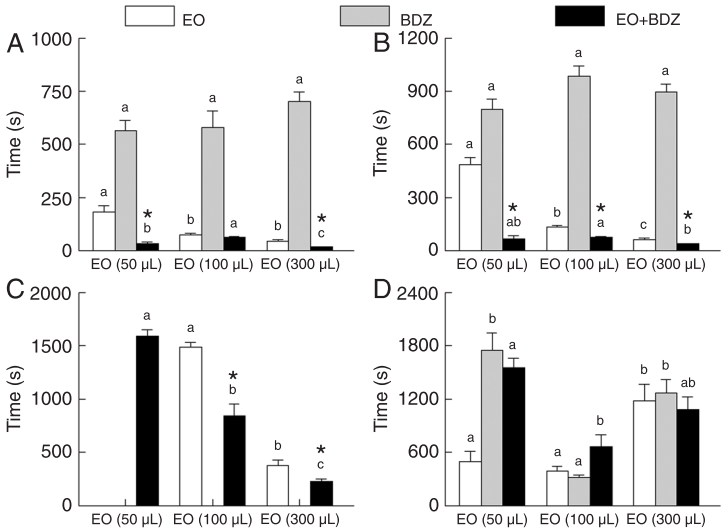

Figure 1. Time required for the induction of and recovery from anesthesia using the essential oil of *Lippia alba* (EO1), diazepam (BDZ) and the essential oil + diazepam (EO+BDZ) combination in silver catfish juveniles. Stages according to Schoettger and Julin (19). Maximum observation time was 30 min. Time to reach different stages of anesthesia: *A*, stage 2 (deep sedation); *B*, stage 3a (partial loss of equilibrium); *C*, stage 4 (deep anesthesia); *D*, recovery time. Different letters indicate significant differences between concentrations in the same group: EO, BDZ or EO+BDZ. *P < 0.05 compared to the EO group tested at the same concentration (one-way ANOVA followed by the Tukey test or Kruskal-Wallis and Mann-Whitney tests).

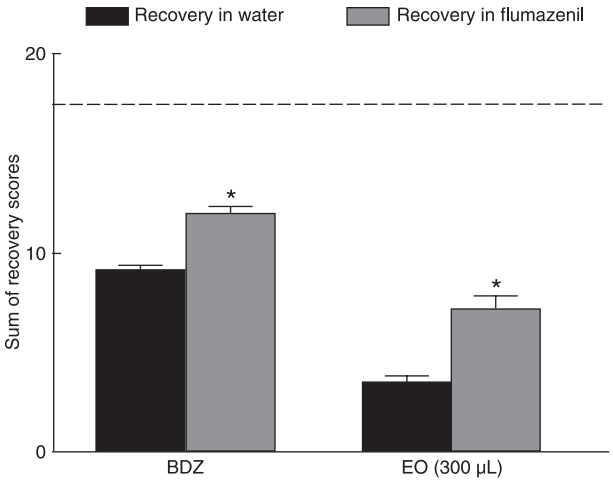

Figure 2. Sum of the recovery scores after anesthesia of *Rhamdia quelen* silver catfish. *P < 0.05 compared to recovery in water (two-way ANOVA and Tukey test). The maximum score possible (17.5) is indicated by the dotted line.

calculations, 100% corresponded to the maximum score possible (17.5). Fish exposed to EO showed percent recovery scores of 40.91% with flumazenil and 20.0% in water. Similarly, for the standard drug (BDZ), we observed percent recovery scores of 68.57% in the presence of flumazenil and of 52.34% under control conditions (water).

Flumazenil, a $GABA_A$ competitive inhibitor, significantly accelerated recovery from BDZ- and EO-induced depression, suggesting the involvement of $GABA_A$ receptors in the anesthetic effect of *L. alba* EO in silver catfish. However, the existence of other possible neurochemical mechanisms, such as anti-nicotinic (27) and anti-glutamatergic (28) activity, cannot be excluded on the basis of the current results.

The literature describes the central nervous system activity of some essential oils, including their effect on the GABAergic system (29-32). Some natural products, such as terpenoids, which are the main components of the EO tested, have exhibited effects on $GABA_A$ receptors (26). According to the literature, some of these constituents, such as α-pinene (33), nerolidol (34), linalool (34,35), borneol (36), and viridiflorol (37) are positive modulators of $GABA_A$ receptors. This result was also observed for EO1 and EO2, which contain these substances, as shown in Table 1. However, given that EOs are complex mixtures of components and that some of their constituents can act by synergism, more studies are necessary to discover the active compound(s) responsible for the anesthetic effect of *L. alba* EO.

We showed for the first time the effect of BDZ on the potentiation of the anesthetic effects of EO in silver catfish. In addition, the anesthetic effects of the EO of *L. alba* were reversed sooner by flumazenil, suggesting the involvement of the GABAergic system in this effect of the EO.

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