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Neuropharmacological effects of essential oil from the leaves of *Croton conduplicatus* Kunth and possible mechanisms of action involved

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ABSTRACT

Ethnopharmacological relevance: *Croton conduplicatus* Kunth (Euphorbiaceae) is a Brazilian aromatic medicinal plant, widely known as “quebra-faca”. In folk medicine, its leaves and stem-barks are used as a natural analgesic for the treatment of headaches.

Aim of the study: In this study, we describe for the first time the neuropharmacological potential of the essential oil obtained from the leaves of *Croton conduplicatus* (EO) in experimental models of pain, anxiety and insomnia. The mechanisms of action involved in these activities were also investigated.

Material and methods: Different experimental models were used to evaluate the antinociceptive (acetic acid, formalin-induced nociception and hot plate tests), anxiolytic (elevated plus maze and hole board tests) and sedative (thiopental-induced sleeping time) effects of EO in mice. EO was evaluated in three different doses (25, 50 and 100 mg/kg, i.p.) and compared with positive and negative controls in all experimental protocols. When appropriate, animals were pretreated with pharmacological antagonists (naloxone, atropine and flumazenil) in order to evaluate the mechanisms of action involved. A docking study also was performed to identify possible targets involved.

Results: EO (25, 50 and 100 mg/kg, i.p.) demonstrated a significant antinociceptive activity in all experimental models. Pretreatment with naloxone or atropine reversed the antinociceptive response ($p < 0.05$), suggesting the involvement of opioid and muscarinic receptors, respectively. A docking study was performed with the major components identified in EO (1,8 cineole − 21.42%, spathulenol – 15.47%, *p*-cymene – 12.41% and caryophyllene oxide – 12.15%), demonstrating favorable interaction profile with different subtypes of muscarinic (M2, M3 and M4) and opioids (delta and mu) receptors. EO also showed anxiolytic (mainly at doses of 25 and 50 mg/kg, i.p.) and sedative (only at the dose of 100 mg/kg, i.p.) effects in mice. These pharmacological responses were reversed by flumazenil ($p < 0.05$), indicating possible involvement of $GABA_A$ receptors.

Conclusion: Our findings support the traditional use of this plant as a natural analgesic and suggest that EO is a multi-target natural product, presenting not only antinociceptive effect but also anxiolytic and sedative activities depending on the dose used.

Abbreviations: ATP, atropine; CNS, central nervous system; DZP, diazepam; EO, essential oil (EO); EPM, elevated plus maze; FLU, flumazenil; GC-MS, gas chromatograph interfaced to a mass spectrometer; INDO, indomethacin; MOR, morphine; NECA, number of entries in the closed arms; NEOA, number of entries in the open arms; NLX, naloxone; TPCA, time of permanence in the closed arms; TPOA, time of permanence in the open arms

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1. Introduction

Central Nervous System (CNS) disorders affect millions of people worldwide. Depressive disorders, dementia, anxiety, insomnia, epilepsy and pain are among the major neuropsychiatric disorders that affect the world population. These clinical conditions are responsible for a significant proportion of the world's disease burden, gradually overcoming the damage attributed to coronary heart disease and cancer (Whiteford et al., 2015; Baxter et al., 2013).

A recent study has shown a considerable decline in the relative proportion of Phase I trial starts for CNS drugs compared with oncology drugs. Furthermore, CNS drugs presented a lower success rate than non-CNS drugs in the Phase III trials. The main reason for discontinuation was the failure to demonstrate efficacy (Kesselheim et al., 2015). Moreover, the drugs used in neurological diseases treatment have a variety of side effects, which reinforces the need to search new bioactive molecules.

Aromatic medicinal plants are widely used to treat neurological disorders. These species are being increasingly studied for obtaining molecules to treat anxiety, insomnia, pain, epilepsy and depression. Normally, aromatic plants produce essential oils commonly used in aromatherapy (Gutiérrez et al., 2014; Perry and Perry, 2006; Koulivand et al., 2013). These oils contain volatile compounds, particularly monoterpenes and sesquiterpenes. Due to their hydrophobic character, terpenoids can easily cross the blood-brain barrier and act on the CNS. Several studies have reported the neuropharmacological properties of essential oils, including antidepressant (Lopes et al., 2011), anxiolytic (Mesfin et al., 2014), anticonvulsant (Almeida et al., 2011), sedative (Silva et al., 2013) and antinociceptive (Lenardão et al., 2016) effects.

Croton conduplicatus Kunth (Euphorbiaceae) is an aromatic medicinal plant popularly known as “quebra-faca” and widely distributed in South America, especially in Brazil. In Northeastern Brazil, leaves and stem-barks of this species are used in folk medicine as natural analgesic (Cartaxo et al., 2010). However, to date, there are no enough reports that can scientifically validate the popular use of this plant as well as there are no reports on the effects of its essential oil for the potential treatment of CNS disorders.

In this paper, we conducted a neuropharmacological investigation of the essential oil from the leaves of *C. conduplicatus* in different experimental models to evaluate the antinociceptive, anxiolytic and sedative activities. In addition, the possible mechanisms of action also were investigated, aided by a docking study.

2. Material and methods

2.1. Plant material

The fresh leaves of *Croton conduplicatus* Kunth were collected in the city of Petrolina (Coordinates: S 09°03′54"; W 40°19′12"), State of Pernambuco, Brazil. A voucher specimen (HTSA2421) was deposited at the Herbário do Trópico Semiárido (HTSA) of the Empresa Brasileira de Pesquisa Agropecuária do Semiárido (EMBRAPA-Semiárido).

2.2. Essential oil extraction

Fresh leaves (100 g) were triturated and submitted to extraction by hydrodistillation in Clevenger-type apparatus for two hours. The oil obtained (0.90 ml) was dried with anhydrous sodium sulfate and filtered, and then stored in a refrigerator at a temperature of 4 °C until the pharmacological and chemical analysis.

2.3. Chemical analysis of the essential oil (EO)

The substances presents in EO were investigated on a Shimadzu QP-2010 gas chromatograph interfaced to a mass spectrometer (GC-MS). The following conditions were used: DB-5MS column Agilent Technologies (30 m × 0.25 mm × 0.25 µm); helium (99.999%) carrier gas at a constant flow of 1.1 ml/min; injection volume of 1.0 µl; injector split ratio of 1:10; injector temperature of 250 °C; electron impact mode at 70 eV; ion-source temperature of 280 °C and transfer line temperature of 260 °C. The oven temperature was programmed from 60 °C, with an increase of 3 °C/min to 240 °C. A mixture of linear hydrocarbons (C_8H_{18}–$C_{20}H_{42}$) was injected under the same experimental conditions. The identification of the constituents in EO was performed by comparing the spectra obtained with those of the equipment database (Wiley 7 lib and Nist 08 lib) and by using the Kovats Index, calculated for each constituent as previously described (Adams, 1995; Dool and Kratz, 1963). The data were acquired and processed with PC with Shimadzu GC-MS Solution software.

2.4. Animals

Adult male Swiss mice (30–40 g) were randomly housed in appropriate cages at 22 ± 2 °C on a 12 h light/dark cycle (lights on at 7:00 a.m.) with access to food and water *ad libitum*. Animals were allowed to have a period of acclimation (48 h) before any experimental protocol. They were used in groups of six animals each (n = 6). All pharmacological tests were carried out by the same visual observers. All experimental protocols and procedures were approved by the Animal Care and Use Committee from Universidade Federal do Vale do São Francisco by number 0011/120215.

2.5. Drug treatments

Animals (n = 6) were submitted to different treatments, all intraperitoneally (i.p.). Negative control group was treated with the vehicle used for EO and standard drugs solubilization (0.9% saline + 10 µl of Tween 80, 10 ml/kg). Positive control group was treated with diazepam (1 or 5 mg/kg), morphine (10 mg/kg) or indomethacin (20 mg/kg) according to protocol. The other groups were treated with EO at doses of 25, 50 and 100 mg/kg in all pharmacological tests. Flumazenil (2.5 mg/kg), naloxone (1.5 mg/kg) and atropine (0.1 mg/kg) were used to evaluate possible mechanisms of action.

2.6. Behavioral screening

Behavioral screening was performed following parameters previously described (Almeida, 2006). This test was used in order to investigate general CNS effects of EO. Groups of six mice were treated with vehicle (negative control) or EO (25, 50 and 100 mg/kg). Animals were observed at 30, 60, 120, 180 and 240 min after the treatment. At the end of each observation, signs of toxicity and the presence of death were also checked.

2.7. Exploratory activity (open field test)

The open field test was used to evaluate the exploratory activity of EO. It was used an open field apparatus (Insight, Brazil), consisting of a circular arena divided into 12 squares of equal area. A 60 W lamp was placed (100 cm above the apparatus) to illuminate the arena. In this model, groups of six mice were treated with vehicle (negative control), EO (25, 50 and 100 mg/kg) or diazepam (1 mg/kg), 30 min before being analyzed in the open field, according to protocol previously described (Almeida, 2006; Costa et al., 2014). After treatment, the following parameters were monitored during 5 min: number of squares crossed with the four paws (spontaneous locomotor activity), immobility time (s), number of grooming behavior (grooming) and number of surveys (rearing).

2.8. Antinociceptive activity

2.8.1. Acetic-acid-writhing-induced nociception

This test was performed according to the protocol described by Koster et al. (1959), with adaptations (Almeida et al., 2013). Acetic acid 0.9% was prepared in saline solution and administered intraperitoneally to all animals (0.1 ml/10 g). Groups of six mice were treated with vehicle (negative control), morphine (10 mg/kg), indomethacin (20 mg/kg) and EO (25, 50 and 100 mg/kg) were administered 30 min before the acetic acid injection. The number of writhings occurring between 5 and 15 min after acetic acid injection was counted to each group. Writhing was defined as a contraction of the abdominal wall, pelvic rotation followed by hind limb extension.

2.8.2. Formalin-induced nociception

In this model, it was used an experimental protocol similar to that described by Hunskaar and Hole (1987). A formalin solution 2.5% (in saline solution) was injected into the right hind paw of the mice (20 µl/paw subplantar). Vehicle (negative control), morphine (10 mg/kg), indomethacin (20 mg/kg) and EO (25, 50 and 100 mg/kg) were administered 60 min before the formalin injection. Animals were observed in chambers with a mirror mounted on three sides and the amount of time (s) spent licking and biting the injected paw was measured as an indicator of pain. Responses were measured for 5 min after formalin injection (first phase, neurogenic) and 15–30 min after formalin injection (second phase, inflammatory). For evaluation of the involvement of opioid and cholinergic receptors, mice were pretreated with naloxone (1.5 mg/kg) and atropine (0.1 mg/kg) 15 min before administration of morphine (10 mg/kg) or EO (100 mg/kg). After 60 min, the animals were observed under the same conditions described above.

2.8.3. Hot plate test

This test was performed to investigate the antinociceptive effect of EO in mice submitted to a thermic stimulus. Animals were placed on hot plate apparatus (Insight, Brazil) at 55 ± 0.5 °C. A pre-selection of the mice was carried out 24 h before the experiments. Animals showing a reaction time (defined as the latency for licking the hind feet or jumping) greater than 20 s were discarded. Selected mice were treated with vehicle (negative control), morphine (10 mg/kg) and EO (25, 50 and 100 mg/kg). Latency time was recorded for each animal on the hot plate (55 °C) during a maximum period of 20 s, at intervals of 30, 60, 90 and 120 min after the treatments (Almeida et al., 2013).

2.9. Anxiolytic activity

2.9.1. Elevated plus maze (EPM) test

The elevated plus-maze apparatus (Insight, Brazil) consists of two open arms and two closed arms elevated to a height of 40 cm above the floor, in a perpendicular position. Groups of six mice were treated with vehicle (negative control), EO (25, 50 and 100 mg/kg) and diazepam (1 mg/kg). Animals were treated 30 min before being placed on the central platform of the apparatus and the following parameters were registered for 5 min: Number of Entries in the Open (NEOA) and Closed (NECA) Arms, Time of Permanence in the Open (TPOA) and Closed (TPCA) Arms (Almeida, 2006; Costa et al., 2014). To evaluate the involvement of GABA/benzodiazepine receptors in the EO anxiolytic effect, animals were pretreated with flumazenil (2.5 mg/kg) 15 min before administration of diazepam (1 mg/kg) or EO (100 mg/kg). After thirty minutes, the animals were analyzed for five minutes under the same conditions described above.

2.9.2. Hole board test

In this model, a hole board apparatus (Insight, Brazil) was used. The apparatus was elevated to a height of 40 cm above floor and consisted of 5 equidistant holes of 3 cm in diameter. Photocells below the surface of the holes measured the number of head-dips. Groups of six animals were treated with vehicle (negative control), EO (25, 50 and 100 mg/kg) and diazepam (1 mg/kg) 30 min before placed on the center of the apparatus. After treatments, all animals were observed for 5 min to record the total number of head-dips and time spent for the first head-dip (latency) (Almeida, 2006; Costa et al., 2014). To evaluate the involvement of GABA/benzodiazepine receptors in the EO anxiolytic effect, animals were pretreated with flumazenil (2.5 mg/kg) 15 min before administration of diazepam (1 mg/kg) or EO (100 mg/kg). After 30 min, the animals were analyzed for five minutes under the same conditions described above.

2.10. Sedative activity (thiopental-induced sleeping time)

Thiopental-induced sleeping time test was performed to evaluate a possible sedative effect of EO. In this test, sleep state was induced with intraperitoneal administration of sodium thiopental (80 mg/kg) 30 min after the animals were submitted to the different treatments (Almeida, 2006; Costa et al., 2014). Groups of six mice were treated with vehicle (negative control), EO (25, 50 and 100 mg/kg) and diazepam (5 mg/kg). Latency (time spent until the animal loses the righting reflex after sleep induction) and time of sleeping (time elapsed between loss and recovery of the righting reflex) were recorded. Time of sleeping of 120 consecutive minutes was used as cutoff value for the animals of all groups. To evaluate the involvement of GABA/benzodiazepine receptors in the EO sedative effect, mice were pretreated with flumazenil (2.5 mg/kg) 15 min before administration of diazepam (5 mg/kg) or EO (100 mg/kg). After 30 min, the animals were analyzed under the same conditions described above.

2.11. Motor coordination evaluation (rota-rod test)

A rota-rod apparatus (Insight, Brazil) was used for the evaluation of motor coordination (Silva et al., 2015). Initially, mice capable of remaining on the rota-rod apparatus longer than 180 s (7 rpm) were selected 24 h before all experiments. Groups of six mice were treated with vehicle (negative control), EO (25, 50 and 100 mg/kg) and diazepam (1 mg/kg). Each animal was individually evaluated on the rota-rod apparatus at 30, 60 and 120 min after treatments, and the time (s) the mice were able to remain on top of the bar was recorded for up to 180 s.

2.12. Docking study

The structure of muscarinic receptors M2 in complex with (3*R*)-1-azabicyclo[2.2.2]oct-3-yl hydroxy(diphenyl)acetate (PDB ID 3UON) (Haga et al., 2012), M3 in complex with tiotropium (PDB ID 4DAJ) (Kruse et al., 2012), M4 in complex with tiotropium (PDB ID 5DSG) (Thal et al., 2016); Delta Opioid Receptor in complex with naltrindole (PDB ID 4N6H) (Fenalti et al., 2014) and Mu Opioid Receptor in complex with methyl 4-{[(5beta,6alpha)-17-(cyclopropylmethyl)-3,14-dihydroxy-4,5-epoxymorphinan-6-yl]amino}-4-oxobutanoate (PDB ID 4DKL) (Manglik et al., 2012) were downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). Structure of majoritary compounds 1,8-cineole, spathulenol, caryophyllene oxide and *p*-cymene were submitted to molecular docking using the Molegro Virtual Docker, v. 6.0.1 (MVD) (Thomsen and Christensen, 2006). All of the water compounds were deleted from the enzyme structure, and the enzyme and compound structures were prepared using the same default parameter settings in the same software package (Score function: MolDock Score; Ligand evaluation: Internal ES, Internal HBond, Sp2-Sp2 Torsions, all checked; Number of runs: 10 runs; Algorithm: MolDock SE; Maximum Interactions: 1500; Max. population size: 50; Max. steps: 300; Neighbour distance factor: 1.00; Max. number of poses returned: 5). The docking procedure was performed using a GRID of 15 Å in radius and 0.30 in resolution to cover the ligand-biding site of the receptors. The Moldock score [GRID] algorithm was used as the score function, and the Moldock search algorithm was used (Thomsen and

Christensen, 2006).

2.13. Statistical analysis

All data obtained were presented as mean ± standard error of the mean (SEM) and the statistical significance was determined using an analysis of variance (ANOVA) followed by Tukey's test or Kruskal-Wallis test followed by Dunn's post-test, according to the case. Values were considered significantly different at $p < 0.05$. All analysis was performed using by GraphPad Prism 6.0 program (Graph Pad Prism Software Inc., San Diego, CA, USA).

3. Results

3.1. Chemical composition of EO

Croton conduplicatus produced a yellow essential oil with characteristic odor. GC-MS analysis showed the presence of 50 distinct peaks, of which 42 were identified, corresponding to 97.2% of the chemical composition identified for EO. The monoterpenes 1,8-cineole (21.42%) and *p*-cymene (12.41%) and sesquiterpenes spathulenol (15.47%) and caryophyllene oxide (12.15%) were considered the major constituents of the sample (Table 1).

3.2. Behavioral screening

In general, animals treated with EO showed signs of CNS depression at all times of analysis, but no deaths were observed. The main behavioral alterations were: hypnosis, palpebral ptosis and decrease in ambulation, grooming and rearing behaviors. These effects were more evident at 30 and 60 min after treatments.

3.3. Exploratory activity (open-field test)

In this test, animals treated with 50 and 100 mg/kg EO showed a decrease of 72% and 85% in the number of crossings (ambulation) and an increase of 219% and 318% in the immobility time, respectively, compared to the negative control group. Similar result was observed for diazepam. This suggests a possible CNS depressant effect in a dose dependent manner, since 25 mg/kg EO did not cause any significant changes in the evaluated parameters (Fig. 1).

During the test, the number of rearing and grooming were also recorded. EO significantly decreased the number of rearing and grooming at the dose of 100 mg/kg. The same was observed at the dose of 50 mg/kg for the number of grooming (data not shown). These findings support the hypothesis that EO exerts a CNS depressing effect in mice.

3.4. Antinociceptive activity

Acetic-acid-writhing-induced nociception test was the first experimental model to evaluate the antinociceptive effect of EO. In this model, EO significantly reduced the number of abdominal writhings induced by acetic acid at all doses, compared to the negative control group (Fig. 2). This effect was stronger in animals treated with 100 mg/kg EO, in which the number of writhings was reduced by 91%. Morphine and indomethacin presented 100% and 98% of antinociceptive activity, respectively.

In the formalin-induced nociception model, EO significantly reduced the paw licking time compared to the negative control group, at all doses and in both phases. In the first phase, EO (25, 50 and 100 mg/kg) presented 27%, 51% and 45% of antinociceptive activity, respectively. In the second phase, EO (25, 50 and 100 mg/kg) decreased licking time by 62%, 63% and 58%, respectively. Indomethacin presented strong antinociceptive effect in the second phase of the test, while morphine showed a strong effect in both phases (Fig. 3).

To evaluate the role of muscarinic receptors in the antinociceptive effect of EO, animals were pretreated with atropine (ATP 0.1 mg/kg, i.p.), a non-selective muscarinic receptors antagonist. According to Fig. 4, animals treated with EO (100 mg/kg, i.p.) presented reduction in licking time ($p < 0.05$), whereas animals pretreated with atropine (EO + ATP group) had no significant effect compared to negative control group in both phases of formalin test. Results demonstrated by EO and EO + ATP groups were also significantly different, suggesting that pretreatment with atropine completely reversed the antinociceptive effect of EO. This is an important evidence of involvement of muscarinic receptors in its antinociceptive effect.

To evaluate the role of opioid receptors in the antinociceptive effect of EO, animals were pretreated with naloxone (NLX 1.5 mg/kg, i.p.), a non-selective opioid receptors antagonist. According to Fig. 5, animals treated with EO (100 mg/kg, i.p.) presented reduction in licking time ($p < 0.05$), whereas animals pretreated with naloxone (EO + NLX

Table 1
Chemical constituents of the essential oil from the leaves of *Croton conduplicatus* Kunth. (Euphorbiaceae).

Peak	RT (min)	RI	Compound	% GC-MS
1	8.447	914	Tricyclene	0.08
2	8.726	922	α-Thujene	0.50
3	8.927	927	α-Pinene	2.30
4	9.465	941	Camphene	0.49
5	10.521	968	Sabinene	1.46
6	11.731	1000	α-Phellandrene	1.44
7	12.574	1022	*p*-Cymene	12.41
8	12.792	1027	1,8-Cineole	21.42
9	13.694	1051	NI	0.07
10	13.942	1057	γ-Terpinene	0.14
11	15.087	1087	Terpinolene	0.05
12	15.569	1099	(*E*)-Sabinene	0.03
13	15.716	1103	NI	0.13
14	16.402	1122	(*Z*)-*p*-Menth-2-en-1-ol	0.16
15	16.535	1125	α-Campholenal	0.01
16	16.977	1137	(*E*)-Pinocarveol	0.18
17	17.117	1141	Camphor	0.32
18	17.842	1161	Pinocarvone	0.09
19	17.989	1165	Borneol	0.52
20	18.130	1168	NI	0.05
21	18.417	1176	Terpinen-4-ol	2.28
22	19.001	1192	α-Terpineol	0.60
23	22.236	1285	Isobornyl acetate	0.32
24	25.167	1373	α-Copaene	0.20
25	25.450	1382	β-Bourbonene	0.21
26	25.713	1390	β-Elemene	0.34
27	26.560	1417	(*E*)-Caryophylene	7.52
28	27.613	1451	α-Humulene	1.55
29	27.841	1458	Alloaromadendrene	1.69
30	28.473	1478	Germacrene D	0.31
31	28.628	1483	β-Selinene	0.32
32	28.955	1494	Bicyclogermacrene	1.61
33	29.488	1512	δ-Amorphene	0.58
34	29.776	1521	δ-Cadinene	0.53
35	30.355	1541	α-Calacorene	0.14
36	30.611	1550	NI	0.32
37	34.413	1577	Spathulenol	15.47
38	31.541	1581	Caryophyllene oxide	12.15
39	32.105	1601	Ledol	1.50
40	32.265	1607	Humulene epoxide	1.42
41	32.450	1613	Cubenol	0.20
42	32.826	1627	Acorenol	0.25
43	32.966	1632	NI	0.44
44	33.069	1636	NI	1.19
45	33.185	1640	Epi-α-Cadinol	4.34
46	33.352	1646	α-Muurolol	0.50
47	33.449	1649	β-Eudesmol	0.51
48	33.578	1654	α-Cadinol	1.02
49	33.881	1665	NI	0.45
50	35.751	1734	NI	0.15
Total identified				**97.2**

RT: retention time of compounds. RI: retention indices on DB-5MS column (relative to *n*-alkanes). NI: not identified compound.

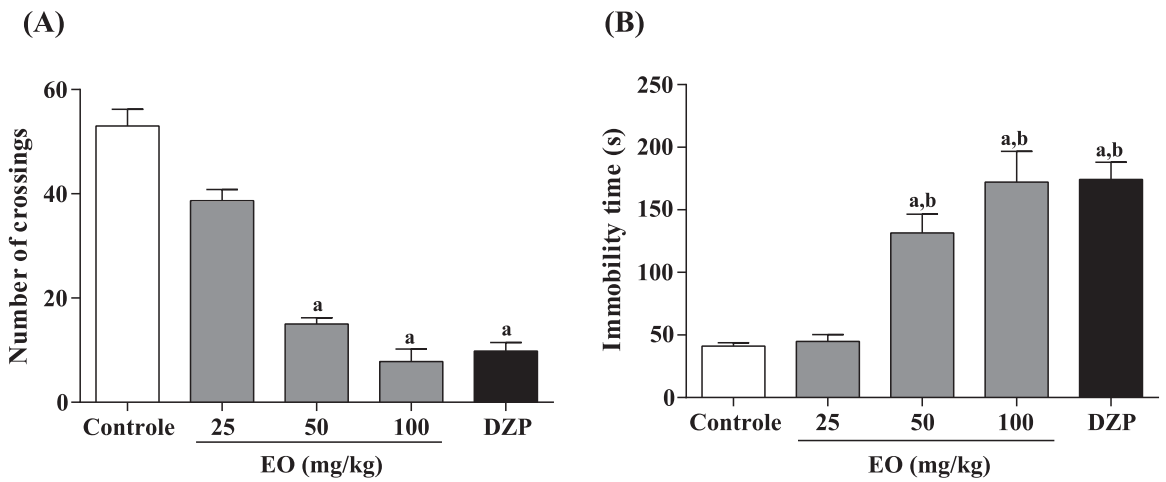

Fig. 1. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and diazepam (DZP 1.0 mg/kg, i.p.) in the open field test, in mice (n = 6, per group). Values are expressed as mean ± SEM, where *a* and *b* indicate $p < 0.05$, significantly different from the control group and EO 25 mg/kg group, respectively, according to the Kruskal Wallis test, followed by the Dunn's post-test (A) or according to ANOVA, followed by the Tukey's test (B).

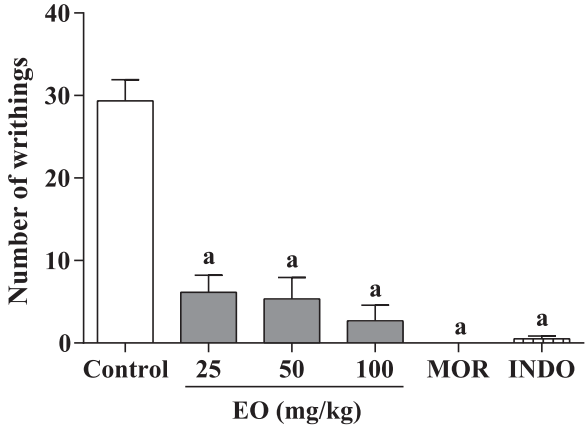

Fig. 2. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.), morphine (MOR 10 mg/kg, i.p.) and indomethacine (20 mg/kg, i.p.) in the acetic-acid-writhing-induced nociception test, in mice (n = 6, per group). Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from the control group, according to the Kruskal Wallis test, followed by the Dunn's post-test.

group) had no significant effect compared to negative control group, but only in the first phase of formalin test, suggesting the involvement of opioid receptors in the central antinociceptive effect of EO. Naloxone significantly reversed the antinociceptive effect of morphine in both phases of the test ($p < 0.05$).

In the hot plate test, EO increased the latency time of the animals on the heated plate only at the highest dose (100 mg/kg, i.p), after 60 min of treatment, confirming that the antinociceptive activity of EO is possibly mediated by central mechanisms such as cholinergic and opioid neurotransmission systems. Morphine presented strong antinociceptive activity at 30, 60 and 90 min after treatment (Fig. 6).

3.5. Anxiolytic activity

In order to evaluate the anxiolytic effect of EO, EPM test was performed. In this model, EO did not change NEOA, but reduced NECA and increased TPOA at all doses. In addition, 50 and 100 mg/kg EO significantly decreased TPCA when compared to the negative control group (Fig. 7). A similar result was observed for animals treated with diazepam, suggesting a possible anxiolytic effect of EO.

To confirm the anxiolytic effect observed in the elevated plus maze test, the hole board assay was performed. In this experimental model, 100 mg/kg EO decreased the time spent for the first head-dip (latency)

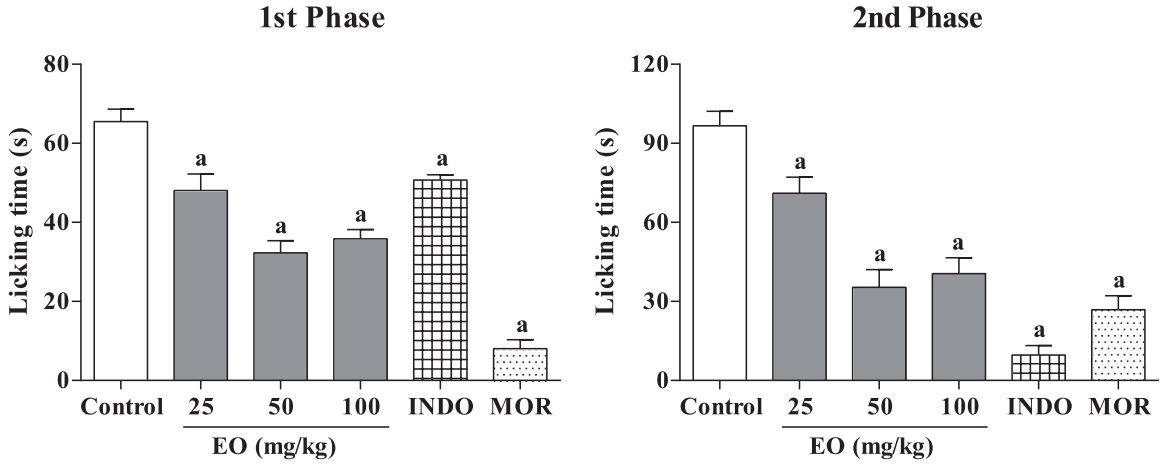

Fig. 3. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.), morphine (MOR 10 mg/kg, i.p.) and indomethacine (20 mg/kg, i.p.) in the first and second phase of formalin-induced nociception test (n = 6, per group). Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from the control group, according to ANOVA, followed by the Tukey's test.

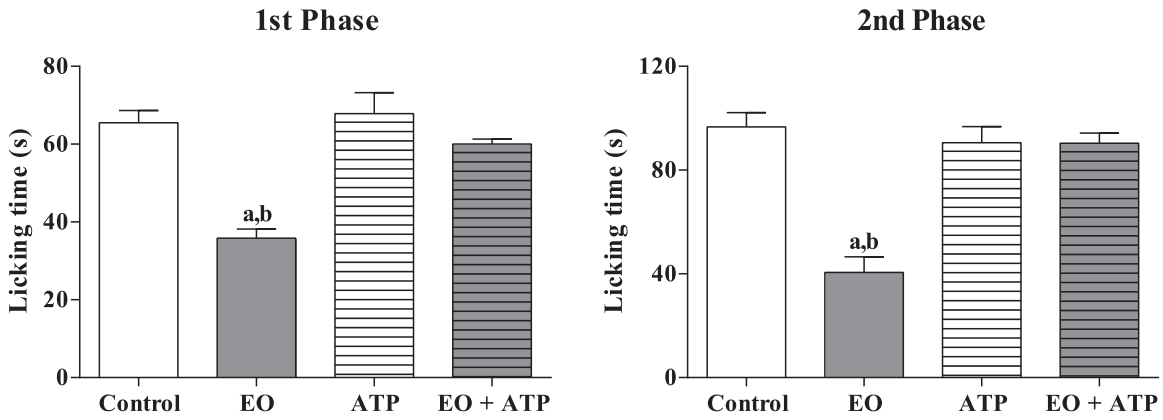

Fig. 4. Effect of the essential oil of *C. conduplicatus* (EO 100 mg/kg, i.p.), atropine (ATP 0.1 mg/kg, i.p.) and EO + ATP (100 and 0.1 mg/kg, respectively, i.p.) in the first and second phase of formalin-induced nociception test (n = 6, per group). Values are expressed as mean ± SEM, where *a* and *b* indicate $p < 0.05$, significantly different from negative control and EO + ATP groups, respectively, according to ANOVA, followed by the Tukey's test.

and increased the total number of head-dips performed by the mice (Fig. 8). Diazepam (1 mg/kg, i.p.) presented the same changes observed for EO, suggesting that the essential oil may act as benzodiazepine.

To evaluate the involvement of $GABA_A$ receptors in the anxiolytic effect of EO, animals were pretreated with flumazenil (FLU 2.5 mg/kg, i.p.), a competitive $GABA_A$ receptors antagonist. In the elevated plus maze test, pretreatment with flumazenil (FLU 2.5 mg/kg, i.p.) reversed the pharmacological response of EO (EO 100 mg/kg, i.p.) in the TPBA and TPBF parameters, suggesting that, at least in part, $GABA_A$ receptors may be involved in its anxiolytic effect (Fig. 9). Similarly, flumazenil also reversed the anxiolytic action of diazepam in all parameters evaluated.

Involvement of the GABAergic system was also investigated in the hole board test. When administrated alone, EO demonstrated relevant anxiolytic effect in all analyzed parameters, decreasing time spent for the first head-dip (latency) and increasing the number of head-dips (Fig. 10). However, when the animals were pretreated with flumazenil (EO + FLU), the anxiolytic effect of EO was reversed, corroborating with the results shown in the elevated plus maze test.

3.6. Sedative activity (thiopental-induced sleeping time)

According to Fig. 11, EO (100 mg/kg, i.p.) decreased latency of sleeping (35%) and extended sleeping time (103%), suggesting a possible sedative activity. The same was observed for the diazepam (2 mg/kg, i.p.) treated group. However, 25 and 50 mg/kg EO did not present any significant changes in relation to the control group, indicating that the essential oil presents sedative effect only in high doses.

The role of $GABA_A$ receptors was also evaluated in the sedative effect of EO. In this model, administration of flumazenil (2.5 mg/kg, i.p.) completely reversed the sedative effect observed for EO, altering latency and total sleeping duration in a statistically significant way (Fig. 12). Similar results were found for diazepam (2 mg/kg, i.p.), confirming that $GABA_A$ receptors are also possibly involved in the sedative effect of EO.

3.7. Motor coordination evaluation (rota-rod test)

To evaluate the effect of EO on the motor coordination of mice, rota-rod test was performed. As shown in Fig. 13, there was no change after EO administration, suggesting that there was no impairment of the animal's motor coordination. In contrast, diazepam (1 mg/kg) decreased the residence time of the animals on the rota-rod, a characteristic behavior of the benzodiazepine drugs in this experimental model.

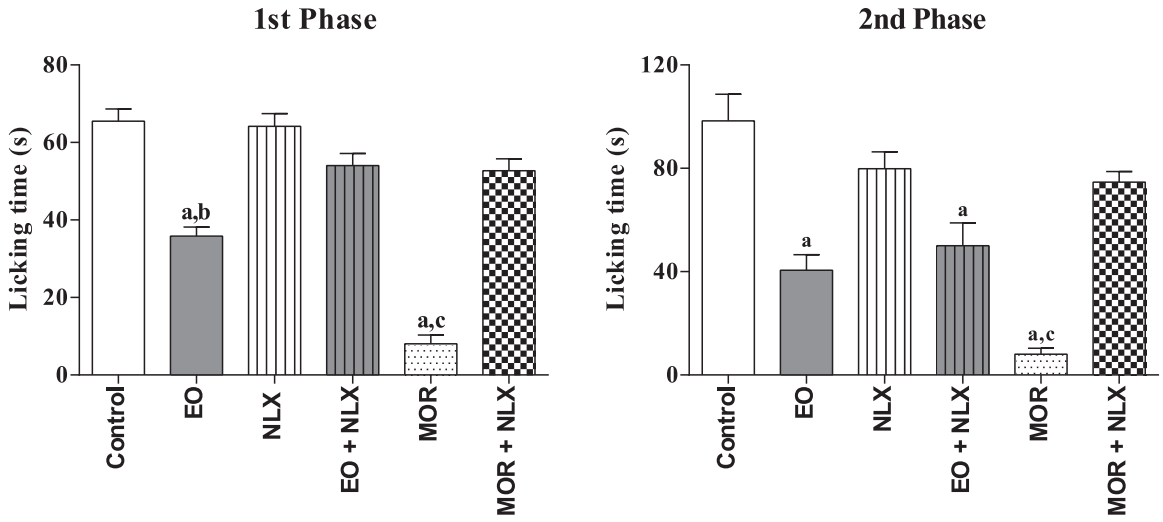

Fig. 5. Effect of the essential oil of *C. conduplicatus* (EO 100 mg/kg, i.p.), morphine (MOR 10 mg/kg, i.p.), naloxone (NLX 1.5 mg/kg, i.p.), EO + NLX (100 and 1.5 mg/kg, respectively, i.p.) and MOR + NLX (10 and 1.5 mg/kg, respectively, i.p.) in the first and second phase of formalin-induced nociception test (n = 6, per group). Values are expressed as mean ± SEM, where *a*, *b* and *c* indicate $p < 0.05$, significantly different from negative control, EO + NLX and MOR + NLX groups, respectively, according to ANOVA, followed by the Tukey's test.

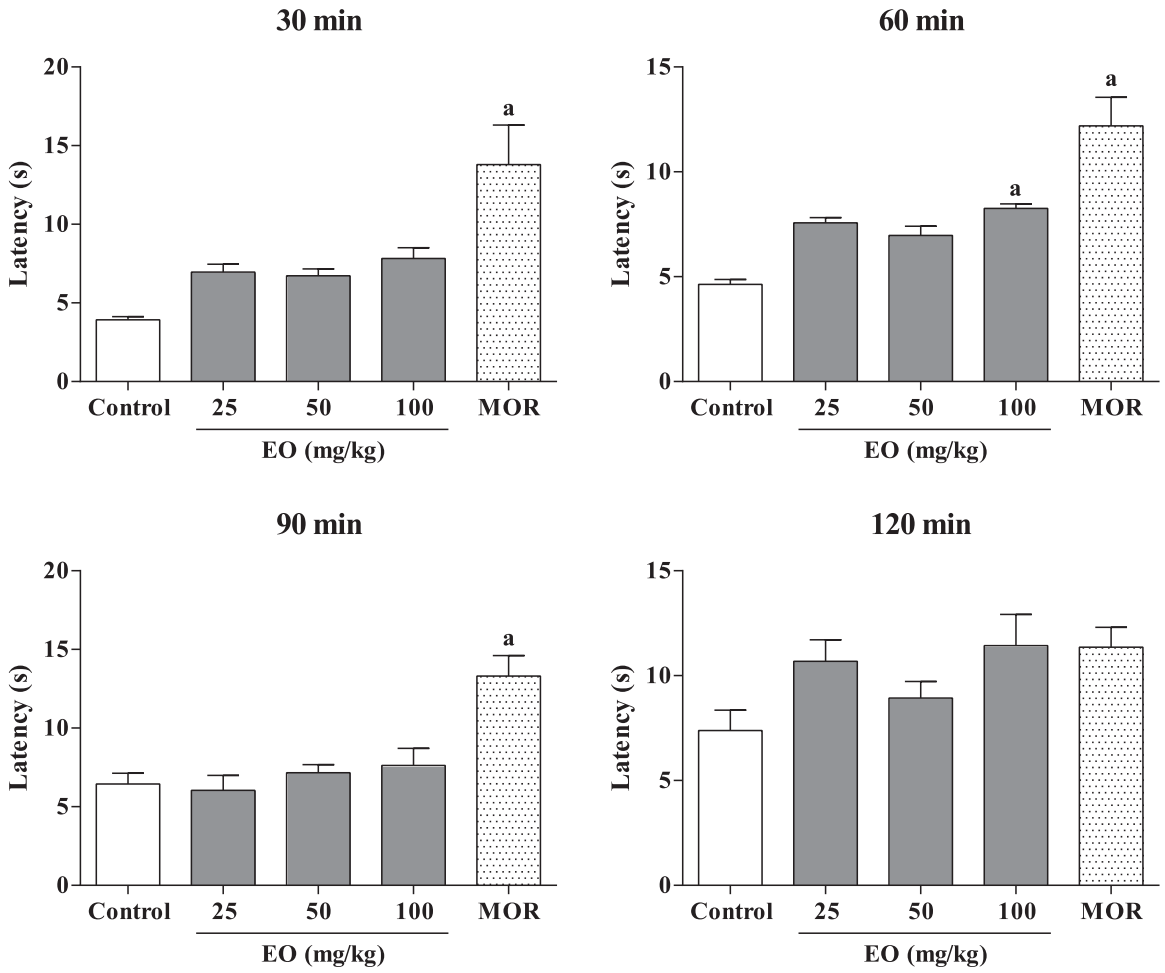

Fig. 6. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and morphine (MOR 10 mg/kg, i.p.) in the hot plate test (n = 6, per group). Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from negative control group, according to ANOVA, followed by the Tukey's test.

3.8. Docking study

Docking was validated redocking the originals ligands in active site of muscarinic receptors M2, M3 and M4 and opioid receptors delta and mu, as observed in crystallography pdb file. The same parameter described to evaluate majoritary compounds of the EO (1,8-cineole, spathulenol, caryophyllene oxide and *p*-cymene) was used in validation. Superpositions of poses are represented in Fig. 14 and shows a good match.

The structure of ligands (3*R*)-1-azabicyclo[2.2.2]oct-3-yl hydroxy (diphenyl)acetate [(*R*)-(-)-3-Quinuclidinyl benzilate (QNB)] co-crystalized with muscarinic receptor M2 and (1R,2R,4S,5S,7S)-7 {[hydroxy (dithiophen-2-yl)acetyl]oxy}-9,9-dimethyl-3-oxa-9-azoniatricyclo [3.3.1.0–2,4~]nonane (tiotropium) co-crystalized with muscarinic receptor M3 and M4, as well as naltrindole co-crystalized with Delta opioid receptor and methyl 4-{[(5beta,6alpha)-17-(cyclopropylmethyl)-3,14-dihydroxy-4,5-epoxymorphinan-6-yl]amino}-4-oxobutanoate co-crystalized with Mu opioid receptor were used in docking performance for energy evaluation. Binding energies of 1,8-cineole, spathulenol, caryophyllene oxide and *p*-cymene even as co-crystalized ligands are show in Table 2.

4. Discussion

GC-MS analysis showed two monoterpenes (1,8-cineole and *p*-cymene) and two sesquiterpenes (spathulenol and caryophyllene oxide) as major constituents of EO. Previous phytochemical investigations have shown these terpenoids as the main components present in essential oils from the fresh leaves of *C. conduplicatus* (Almeida et al., 2015a), corroborating our results and indicating that they are possibly chemical markers of this species.

Initially, a behavioral pharmacological screening was performed as well as open field test to investigate the effect of EO on the exploratory locomotion of the animals. Signs of CNS depression were observed in both experimental protocols (Fig. 1), especially at the highest doses tested (50 and 100 mg/kg, $p < 0.05$).

The antinociceptive effect of EO was evaluated in three different experimental models. First, EO significantly reduced the nociceptive response induced by intraperitoneal administration of acetic acid solution (Fig. 2), exhibiting similar efficacy to standard drugs (morphine and indomethacin). This is a classic model for the evaluation of new analgesic drugs. However, it is a rather specific test, since the administration of the chemical agent promotes the sensitization of peripheral nociceptors, besides inducing the release of mediators involved in neurogenic (serotonin, substance P, dopamine, acetylcholine, noradrenaline) and inflammatory (histamine, bradykinin, prostaglandins, nitric oxide) processes of pain (Gawade, 2012). In this sense, to better characterize the antinociceptive effect of EO, we performed the formalin test.

EO showed significant antinociceptive effect in both phases of the formalin test. This experimental model allows to evaluate the efficacy of a sample in reducing the nociceptive response in the neurogenic and inflammatory phases, separately (Hunskaar and Hole, 1987). In general, molecules that reduce nociceptive response through central

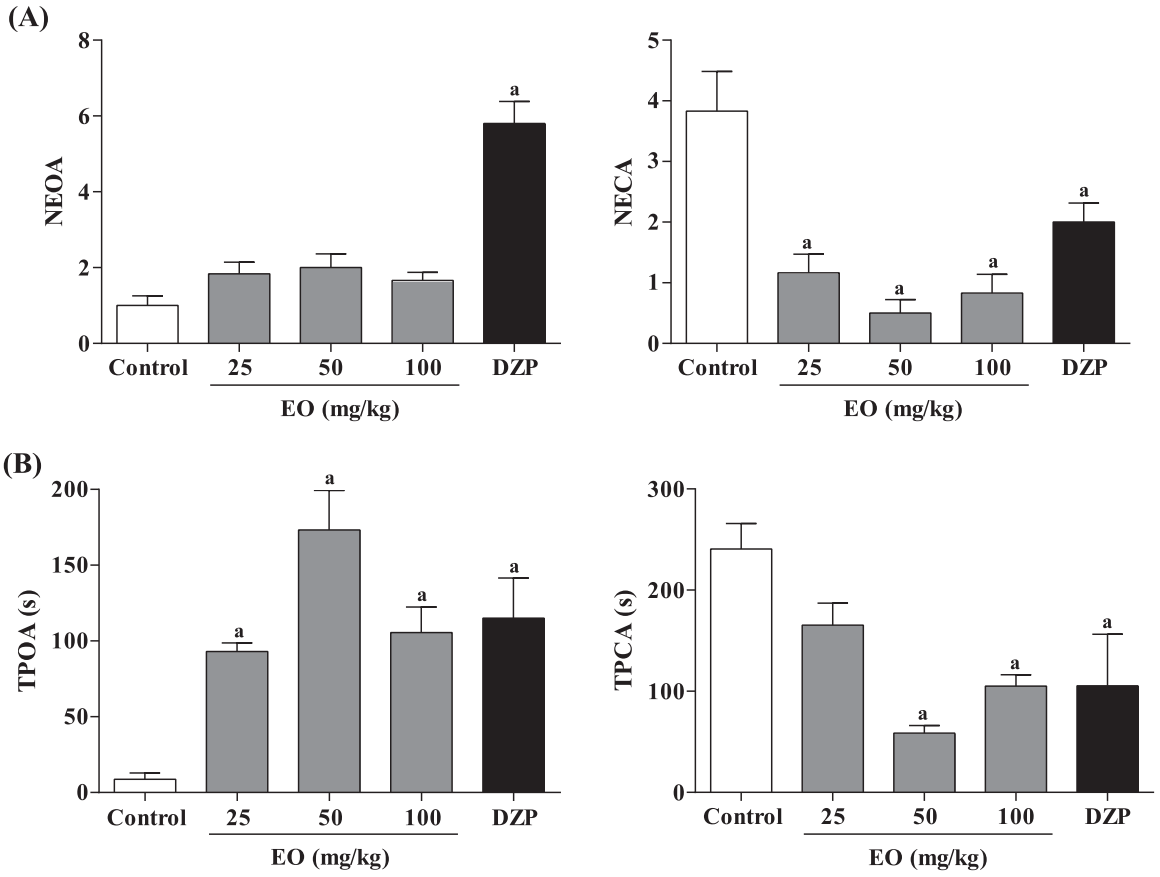

Fig. 7. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and diazepam (DZP 1.0 mg/kg, i.p.) in the elevated plus maze test, in mice (n = 6, per group). NEOA: Number of Entries in the Open Arms, NECA: Number of Entries in the Closed Arms, TPOA: Time of Permanence in the Open Arms, TPCA: Time of Permanence in the Closed Arms. Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from the control group, according to the Kruskal Wallis test, followed by the Dunn's post-test (A) or according to ANOVA, followed by the Tukey's test (B).

antinociceptive mechanisms exhibit strong pharmacological effects in both phases of the test, such as morphine (Fig. 3). Therefore, EO would be acting similarly to morphine, reducing nociceptive behavior by interfering with central mechanisms of pain. This hypothesis was confirmed by the hot plate test, in which EO was able to increase the latency to the nociceptive behavior when the animals were submitted to thermal stimulation (Fig. 6).

Considering the biphasic character of the formalin test, we decided to evaluate the possible mechanisms involved in the antinociceptive effect of EO using this experimental protocol, with the aid of the pharmacological antagonists naloxone and atropine. When animals were pre-treated with naloxone, the antinociceptive effect of EO (100 mg/kg, i.p.) was reversed in the first phase of the test (Fig. 5), suggesting the involvement of opioid receptors at least in part. Meanwhile, pre-treatment with atropine completely reversed the antinociceptive response of EO (100 mg/kg, i.p.) in both phases of the formalin test (Fig. 4), indicating possible involvement of muscarinic receptors. However, naloxone and atropine are non-selective

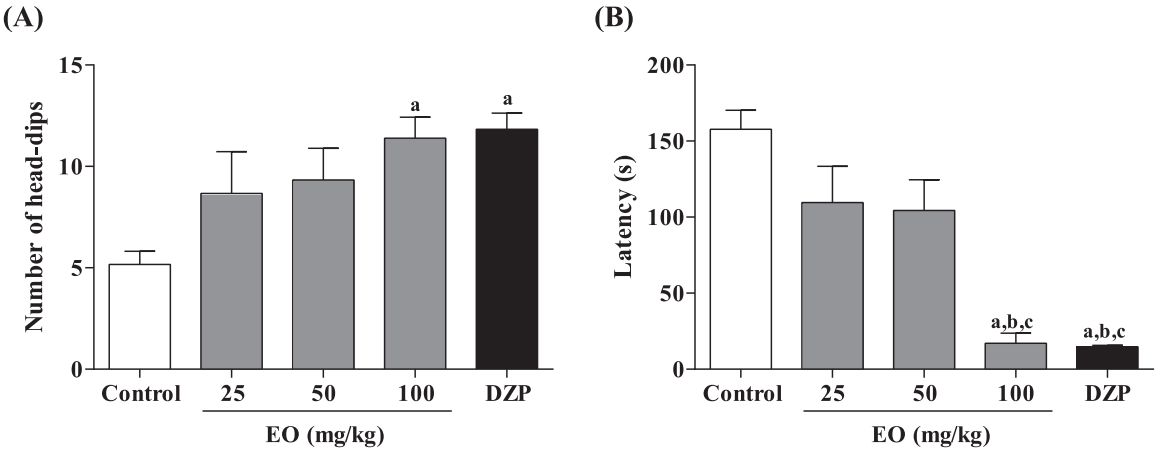

Fig. 8. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and diazepam (DZP 1.0 mg/kg, i.p.) in the hole board test, in mice (n = 6, per group). Values are expressed as mean ± SEM, where *a*, *b* and *c* indicate $p < 0.05$, significantly different from negative control, EO 25 mg/kg and EO 50 mg/kg groups, respectively, according to the Kruskal Wallis test, followed by the Dunn's post-test (A) or according to ANOVA, followed by the Tukey's test (B).

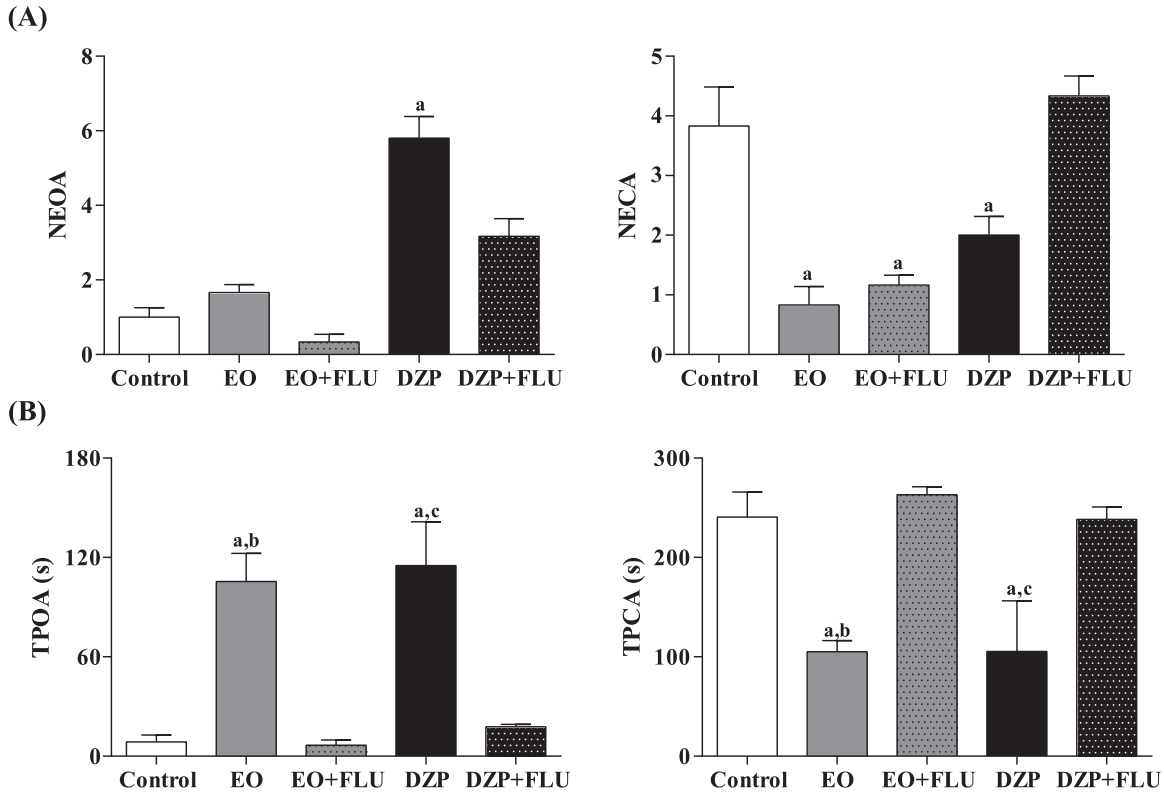

Fig. 9. Effect of the essential oil of *C. conduplicatus* (EO 100 mg/kg, i.p.), diazepam (DZP 1 mg/kg, i.p.), EO + FLU (100 and 2.5 mg/kg, respectively, i.p.) and DZP + FLU (1 and 2.5 mg/kg, respectively, i.p.) in the elevated plus maze test (n = 6, per group). Values are expressed as mean ± SEM, where *a*, *b* and *c* indicate $p < 0.05$, significantly different from negative control, EO + FLU and DZP + FLU groups, respectively, according to the Kruskal Wallis test, followed by the Dunn's post-test (A) or according to ANOVA, followed by the Tukey's test (B).

pharmacological antagonists, and it is not possible to predict which subtypes of EO receptors would be interacting. In this context, we investigated the interaction profile of the major components identified in EO with the main subtypes of opioid and muscarinic receptors involved in pain signaling pathways.

Evaluating binding energy values of four majoritary terpenes from EO, spathulenol (15.47% GC-MS) and caryophyllene oxide (12.15% GC-MS), present better performance in comparison with 1,8 cineole (21.42% GC-MS) and *p*-cymene (12.41% GC-MS), contributing for biological activity observed once spathulenol and caryophyllene oxide represent more than a quarter of EO composition.

Although spathulenol and caryophyllene oxide show better energy values between terpenes evaluated through *in silico* approach, its value is still lower than the ligands used as standard. However, binding energy values allow suggest a synergy effect specially between spathulenol and caryophyllene oxide and a multitarget effect, once it presents a nice interactivity in both muscarinic and opioid receptors (Table 2).

Recently, our research group demonstrated the antinociceptive effect of the essential oil obtained from the stem-barks of *C. conduplicatus* (Oliveira-Júnior et al., 2017). In this study, the involvement of muscarinic receptors (M2, M3 and M4) was also observed. Interestingly, there was no involvement of opioid receptors. This change can be attributed to variations in the chemical composition of the essential oil produced by different parts of the plant (leaves and stem-barks), as previously reported (Oliveira-Júnior et al., 2017; Almeida et al., 2015b).

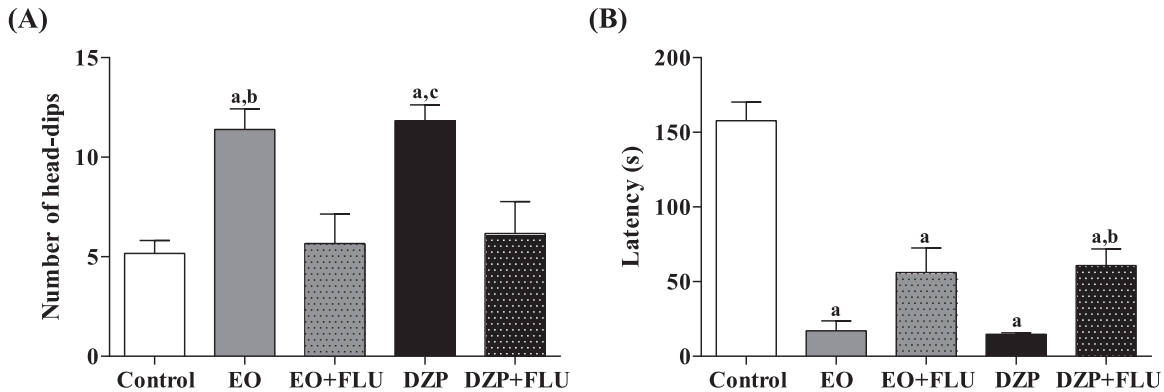

Fig. 10. Effect of the essential oil of *C. conduplicatus* (EO 100 mg/kg, i.p.), diazepam (DZP 1 mg/kg, i.p.), EO + FLU (100 and 2.5 mg/kg, respectively, i.p.) and DZP + FLU (1 and 2.5 mg/kg, respectively, i.p.) in the hole board test (n = 6, per group). Values are expressed as mean ± SEM, where *a*, *b* and *c* indicate $p < 0.05$, significantly different from negative control, EO + FLU and DZP + FLU groups, respectively, according to the Kruskal Wallis test, followed by the Dunn's post-test (A) or according to ANOVA, followed by the Tukey's test (B).

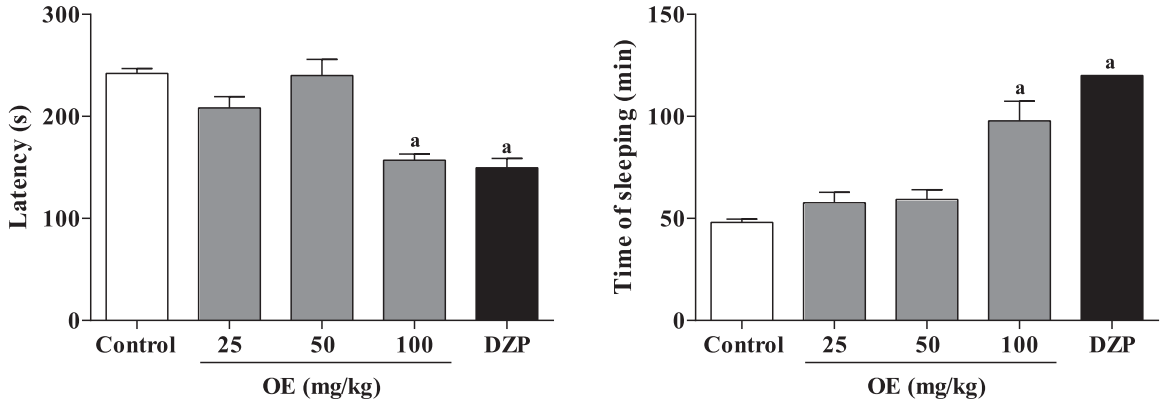

Fig. 11. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and diazepam (DZP 2 mg/kg, i.p.) in the thiopental-induced sleeping time test (n = 6, per group). Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from negative control group, according to ANOVA, followed by the Tukey's test.

In our continuous search for medicinal plants with activity on CNS, we also decided to investigate the anxiolytic and sedative effects of EO in different experimental models. Elevated plus maze is one of the most important animal models used in the assessment of new anxiolytic drugs. In general, anxiolytic agents increase the frequency of entries and the time spent in open arms of EPM. In this sense, EO increased TPOA and reduced TPCA and NECA at all doses, suggesting a relevant anxiolytic effect (Fig. 7). This pharmacological response was confirmed in the hole-board test, in which EO (100 mg/kg, i.p.) significantly reduced latency and increased the number of head-dips (Fig. 8). Usually, drugs as benzodiazepines increase the number of head-dips in the hole-board test, even when evaluated at lower doses (Netto et al., 2009).

When the animals were subjected to the thiopental-induced sleeping time test, EO was able to decrease latency and extend sleeping time only at the highest dose (100 mg/kg, i.p.). This result indicates that EO has a dual effect on the CNS of mice, acting as anxiolytic at low doses (25–50 mg/kg) and sedative at high dose (100 mg/kg), similar to several benzodiazepines conventionally used for treatment of neurological disorders. In this perspective, animals were treated with flumazenil, a $GABA_A$ competitive antagonist, before EO administration (100 mg/kg, i.p.). Figs. 9, 10 and 12 showed that flumazenil reversed the pharmacological response of EO in some important behavioral parameters, suggesting the possible involvement of $GABA_A$ receptors. These findings are in agreement with several pharmacological reports demonstrating that the sedative and anxiolytic potential of monoterpenes and sesquiterpenes appears to be mediated by GABA/benzodiazepine transmission (Granger et al., 2005; Rivera et al., 2014; Milanos et al., 2017; Sousa et al., 2015). Additionally, EO promoted no alteration in the motor coordination of the animals, being considered a satisfactory candidate for the development of new herbal medicines acting on CNS disorders.

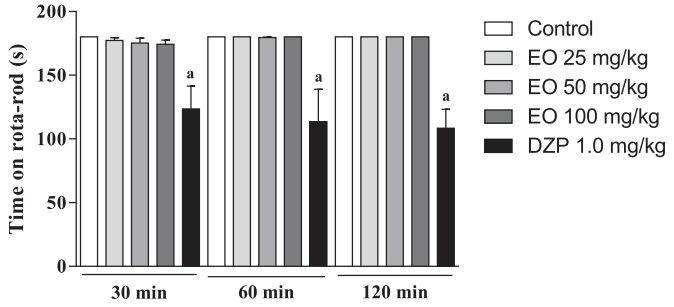

Fig. 13. Effect of the essential oil of *C. conduplicatus* (EO 25, 50 and 100 mg/kg, i.p.) and diazepam (DZP 1 mg/kg, i.p.) in the rota-rod test (n = 6, per group). Values are expressed as mean ± SEM, where *a* indicates $p < 0.05$, significantly different from negative control group, according to ANOVA, followed by the Tukey's test.

5. Conclusions

In summary, EO showed antinociceptive, anxiolytic and sedative effects in different experimental models, without interfering in the motor coordination of the animals. At first, the pharmacological effects observed may vary depending on the dose used and appear to involve muscarinic, opioid and $GABA_A$ receptors, suggesting that EO is actually a multi-target natural product. Additionally, the results described in this study support the popular use of the species for the treatment of pain disorders.

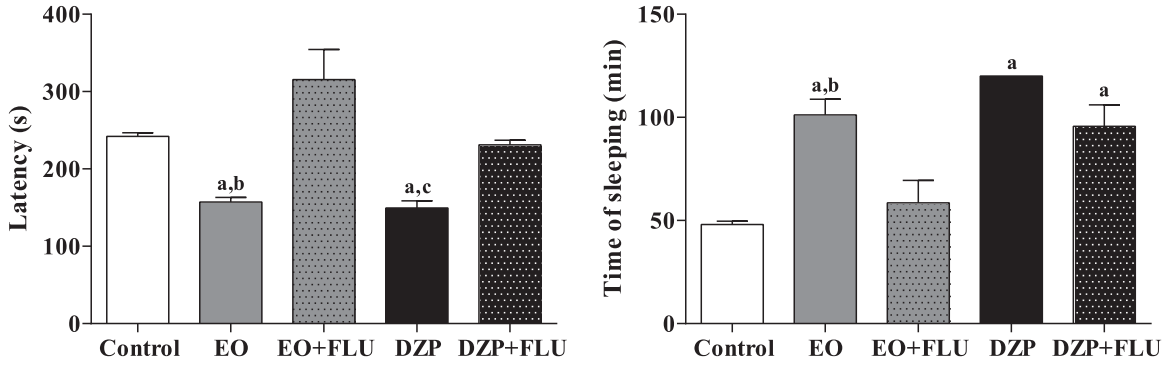

Fig. 12. Effect of the essential oil of *C. conduplicatus* (EO 100 mg/kg, i.p.), diazepam (DZP 2 mg/kg, i.p.), EO + FLU (100 and 2.5 mg/kg, respectively, i.p.) and DZP + FLU (2 and 2.5 mg/kg, respectively, i.p.) in the thiopental-induced sleeping time test (n = 6, per group). Values are expressed as mean ± SEM, where *a*, *b* and *c* indicate $p < 0.05$, significantly different from negative control, EO + FLU and DZP + FLU groups, respectively, according to ANOVA, followed by the Tukey's test.

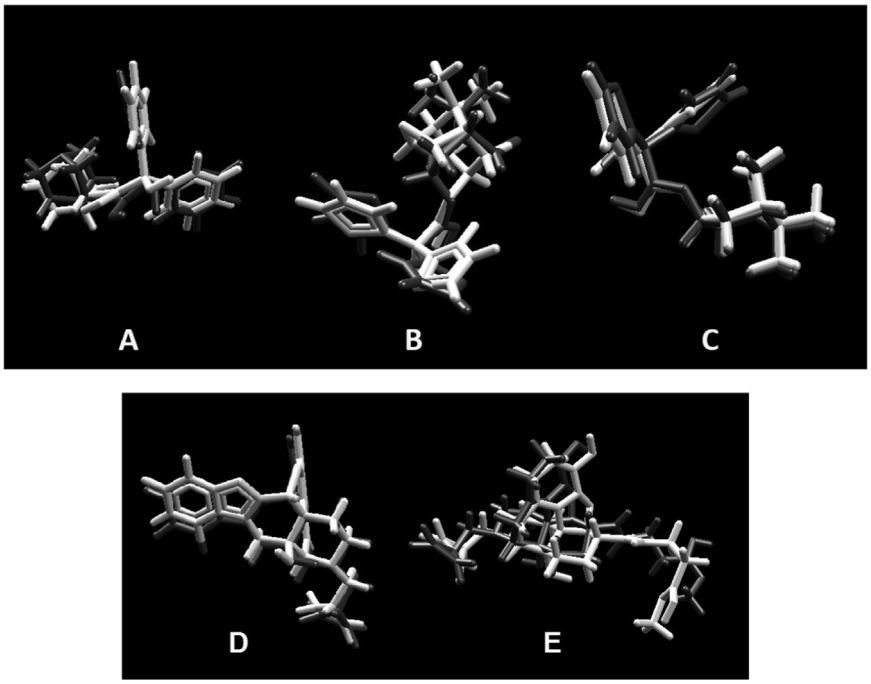

Fig. 14. Superposition of crystal pose (white) and docking pose (gray) validating the methodology. (A) Muscarinic Receptors M2, (B) Muscarinic Receptors M3, (C) Muscarinic Receptors M4, (D) Delta Opioid Receptor and (E) Mu Opioid receptor.

Table 2
MolDock Score energies of majoritary compounds of EO and original ligands on muscarinic receptors M2 (3UON), M3 (4DAJ) and M4 (5DSG) and opioid receptors delta (4N6H) and mu (4DKL).

Compounds	3UON	4DAJ	5DSG	4N6H	4DKL
1,8-Cineole	− 62.6	− 64.4	− 69.1	− 49.4	− 56.5
Caryophyllene oxide	− 110.9	− 114.8	− 119.3	− 98.3	− 97.0
p-Cymene	− 67.2	− 70.6	− 71.8	− 63.5	− 63.7
Spathulenol	− 107.7	− 105.8	− 108.3	− 98.0	− 90.9
QNB	− 149.5	− 146.6	− 146.3	–	–
Tiotropium	− 162.9	− 167.6	− 177.8	–	–
Naltrindole	–	–	–	− 139.7	–
BF0	–	–	–	–	− 109.9

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Authors contributions

Conceived and designed the experiments: JRGSA and RGOJ. Plant collection and essential oil extraction: USS and AVVS. Chemical analysis: MJS and CEPN. Docking study: VPL. Pharmacological experiments: RGOJ, CAAF, JCS, RBAT, MGS and TCD. Data analysis: RGOJ, MGS, TCD and JRGSA. Contributed reagents/materials/analysis tools: JRGSA and LJQJ. Wrote the paper: RGOJ, VPL and CAAF. Design conception and paper review: JRGSA and LJQJ.

Declaration of interest

All authors agree on the content of the manuscript and declare no conflict of interest.

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