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A New B-Triketone and Antinociceptive Effect from the Essential Oil of the Leaves of *Calyptranthes restingae* Sobral (Myrtaceae)

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Abstract

Calyptranthes restingae Sobral, known as "murta", is a species of the northeastern Brazilian used to treat fever, pain and inflammatory disorders. The essential oil from *Calyptranthes restingae* Sobral (Myrtaceae) collected in Sergipe, northeastern Brazil, was obtained through the hydrodistillation and its antinociceptive properties were evaluated. Chromatographic analysis revealed 14 components, but only five were identified, accounting for 98.50% of the oil: (E)-caryophyllene (2.40%), calyptrantone (81.03%), *α*-humulene (1.52%), *β*-selinene (8.54%), *α*-selinene (5.01%). The structure of the major compound, calyptrantone, was elucidated by ^{1}H and ^{13}C-NMR. This is the first time that a *β*-triketone has been identified in the essential oil of *Calyptranthes* genus and that calyptrantone has been reported as a natural product. This essential oil showed an antinociceptive effect in mice, reducing acetic-acid-induced abdominal writhing significantly in comparison with the control group ($p<0.001$), with the possible participation of opioids. In the formalin test, the oil also caused significant inhibition of licking time in both phases without loss of motor coordination. Besides, all doses of the oil decreased the leukocyte migration in peritoneal cavity induced by carrageenan ($p<0.01$). Together, these results indicate that the essential oil of *C. restingae* represents an important potential tool for management of neurogenic and inflammatory pain.

Keywords: Myrtaceae; *Calyptranthes restingae*; *β*-triketone; Calyptrantone; Pain; Inflammation

Introduction

Myrtaceae is one of the largest families of the Brazilian flora, with approximately 100 genera and 3,000 species. It is also one of the most complexes taxonomically, due to the number of species and the scarcity of taxonomic studies [1]. In the Americas, the family is represented mainly by fruiting plants such as *Syzygium malaccensis* ("jambo"), *Psidium guajava* ("guava" or "goiaba" in Brazil), and *Eugenia uniflora* ("pitanga"), which represent only a small fraction of the economic potential of the family, given the large number of non-commercial species that produce edible fruits [2,3].

From a pharmacological perspective, the essential oils of myrtles are widely used in the production of drugs [4,5]. There are a number of applications for the treatment of ulcers, gastritis, leukemia, hypoglycemia, rheumatism, gout and hypotension [6-8], viruses [9] and microbes [10]. Recently, the antinociceptive and hypothermic effects of the essentials oils of *Eugenia uniflora* [11] and *E. candolleana* DC were confirmed in rodents [12].

The American genus *Calyptranthes* comprises about 100 species distributed from Mexico to Uruguay. Most of the phytochemical studies about this genus have been on the chemical composition of the essential oils [13-21]. *Calyptranthes restingae* Sobral, known as "murta", is a rare species of the northeastern Brazilian rainforests. In the Brazilian Northeastern folk medicine, the infusion of fresh leaves is used to treat fever, pain and inflammatory disorders. As part of our interest in the Myrtaceae concerning the potential medical applications, this paper reports the chemical composition of the essential oil from the fresh leaves of *C. restingae*, its anti-nociceptive properties in rodents and the structural elucidation of a new *β*-triketone, named calyptrantone by means of spectroscopic techniques. To the best of our knowledge, there are no previous reports on the chemical composition and biological activity of this species.

Materials and Methods

Plant material

Leaves of *Calyptranthes restingae* Sobral (Myrtaceae) were collected in a "restinga" (sandy coastal vegetation) near Pomonga River (satellite positioning: S 10.47.325/W 36.58.414), in the municipality of Santo Amaro das Brotas, state of Sergipe, Brazil, in January 2008. The voucher specimen (Ribeiro AS, Machado SMF, Passos LO, No. 582) was deposited at the herbarium of the Department of Biology, Federal University of Sergipe.

Isolation of essential oil

The essential oil from the fresh leaves was obtained through hydrodistillation for 3 h using a Clevenger-type apparatus. The oil was physically separated from the water, dried over anhydrous sodium sulphate and filtered. Samples of the oil were transferred to amber glass

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bottles and stored in a freezer until GC analysis. The distillations were performed in triplicate.

GC-MS and GC-FID analysis

GC-MS analyses were carried out using a Shimadzu QP5050A system (Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20i auto sample and J and W Scientific DB-5MS (Folsom, CA, USA) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). Helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL/min and an injection volume of 0.5 µL of a solution sample was employed with injector temperature of 250°C (split ratio of 1:83), and ion-source temperature of 280°C. The oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of 4°C /min to 200°C, then 10°C /min to 300°C, ending with a 10-min isothermal at 300°C. Mass spectra were taken at 70 eV, with a scan interval of 0.5 s and fragments from 40 to 550 Da.

GC-FID analysis was performed using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) apparatus, under the following operational conditions: ZB-5MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) from Phenomenex (Torrance, CA, USA), under the same GC-MS temperature program as above. The percent composition of each component was determined from the area of the component divided by the total area of all components isolated under these conditions, without the use of correction factors and arranged in order of GC elution.

The retention indices were obtained by co-injecting the oil sample with a C_9-C_{18} linear hydrocarbon mixture and calculated according to Van den Dool and Kratz equation. The volatile components were analyzed by means of GC-FID and GC-MS, and identification was made from the comparison of retention indices as well as from the computerized matching of the acquired mass spectra with those stored in the NIST and WILEY mass spectral library of the GC-MS data system and other published mass spectra [22].

Characterization of calyptrantone isolated from *C. restingae*

The high resolution mass spectra were measured in a Shimadzu-model LCMS-IT-TOF (225-07100-34) mass spectrometer. The optical rotations were measured in a Perkin-Elmer 341 digital polarimeter and infra-red spectra were recorded using a Perkin-Elmer FT-IR 1000 spectrometer. Melting points were measured in a Toledo FP90 digital Mettler apparatus and are uncorrected. The ^{1}H-, and ^{13}C-NMR spectra and 2D experiments were obtained on a Bruker ARX-400 NMR spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm), with the coupling constants (J) reported in Hertz (Hz). Deuterated chloroform ($CDCl_3$) was used as solvent and tetrametilsilane (TMS) as the internal standard.

Spectral data of calyptrantone

Calyptrantone (1) [4-acetyl-5-hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione] was obtained as a pale yellow oil; UV (MeOH) λ_{max} 273 e 243 nm; IR (KBr) ν_{max} 3757, 3679, 3653, 3617, 3443, 3328, 3320, 3198, 3075, 3006, 2945, 2866, 2717, 2639, 2499, 2369, 2333, 1730, 1678, 1564, 1477, 1423, 1364, 1350, 1215, 1171, 1049, 961, 938, 874, 839, 787, 725 e 646 cm^{-1}; HRESIMS *m/z* 223.0915 [M-H]; EIMS *m/z* 224[M]$^+$ (100%), 70 and 154; and secondary *m/z* 55, 96, 111, 126, 139, 167, 181, 181, 196, 209; ^{1}H-NMR (400.21 MHz, $CDCl_3$/TMS): δ_H 18.26 (1H, s, $H_{quelated}$); 2.61 (3H, s, H-8); 1.46 (6H, s, H-9 and H-10); 1.37 (6H, s, H-11 and H-12); ^{13}C-NMR (100.6 MHz, $CDCl_3$/TMS): δ_C 210.1 (C-1); 201.7 (C-7); 199.2 (C-5); 196.8 (C-3); 109.4 (C-4); 56.8 (C-2); 52.0 (C-6); 27.4 (C-8); 24.4 (C-9 and C-10); 23.9 (C-11 and C-12).

Pharmacological activity

Animals: Male Swiss mice (26 ± 3 g) were obtained from our research colony and were maintained at a controlled room temperature (21 ± 2°C) with food and water ad libitum, and a 12 h light/12 h dark cycle. Experimental protocols and procedures were approved by the Federal University of Sergipe Animal Care and Use Committee (CEPA/UFS Nº 43/06).

Acetic acid induced writhing: Muscular contractions were induced by intraperitoneal injection (i.p.) of a 0.85% solution of acetic acid (0.1 ml/10 g) as described by Koster et al. [23]. The number of muscular contractions was counted for 15 min after the injection and the data represent the average number of writhes observed. EOCR in doses of 25, 50 and 100 mg/kg (i.p., n=10, per group), the reference drug, morphine (3 mg/kg), and the vehicle (saline + Tween-80 0.2%) were administered intraperitoneally to different groups of the mice 0.5 h before the acetic acid injection. An additional group was pretreated with 1.5 mg/kg of naloxone (i.p.), a nonselective opioid antagonist, 15 min before the i.p. administration of the vehicle (control), EOCR (100 mg/kg), or morphine (3 mg/kg). Subsequently, the acetic-acid-induced writhing test was performed as described above.

Formalin test: The formalin test was carried out as described by Hunskaar and Hole [24]. The animals were treated with the vehicle, EOCR (25, 50, and 100 mg/kg, i.p.), or the reference drug (Aspirin 200 mg/kg, i.p.) 0.5 h before the formalin injection. The observation chamber was a glass box of 30 cm diameter on an acrylic transparent plate floor. Beneath the floor, a mirror was mounted at a 90°C angle to allow clear observation of the paws of the animals. 20 µl of a 1% formalin solution was injected into the dorsal surface of the left hind paw. Each animal was then placed in the chamber and the time spent by the animal licking the injected paw was considered to be a measure of pain. Two distinct phases of intensive licking activity were identified: an early acute phase and a late or tonic phase (0-5 and 15-30 min after formalin injection, respectively).

Evaluation of motor activity: In order to investigate whether the treatments influence the motor activity of the animals and consequently impair the assessment of the nociceptive behavior in the experimental models, the animals' motor activity was evaluated in a rota-rod apparatus [24]. Initially, the mice able to remain on the apparatus (AVS®, Brazil) for more than 180 s (7 rpm) were selected 24 h before the test. The animals selected were then divided into four groups and treated i.p. with the vehicle, EOCR (25, 50, and 100 mg/kg, i.p.), and diazepan (DZP, 1.5 mg/kg). Each animal was tested on the rota-rod apparatus and the time they remained on the bar (up to 180 s) was recorded after 0.5 h.

Leukocyte migration to the peritoneal cavity: Leukocyte migration was induced by the injection of carrageenan (1%, i.p., 0.25 mL) into the peritoneal cavity of the mice 0.5 h after the administration of EOCR (25, 50 and 100 mg/kg, i.p.), dexamethasone (2 mg/kg, s.c.) or the vehicle, with a modification in the technique described by Matos et al. [25]. The animals were anesthetized with sodium pentobarbital (50 mg/Kg, i.p.) and were euthanized by cervical dislocation 4 h after the injection of carrageenan. Shortly afterwards, saline containing EDTA (1 mM, i.p., 3 mL) was injected. A brief massage was immediately applied for the further collection of fluid, which was centrifuged (5,000 rpm, 5 min) at room temperature. The supernatant was discarded and 1 mL of PBS was introduced to the precipitate. An aliquot of 10 µL from this suspension was dissolved in 200 µL of Turk solution and the total number of cells was counted in a Neubauer chamber, under optical

microscopy. The results were expressed as the number of leukocyte/mL.

Statistical analysis

The data obtained were evaluated using Kolmogorov-Smirnov test to verify the normal distribution of variables, one-way analysis of variance (ANOVA) followed by Dunnett's or Fisher's test. In all cases, differences were considered significant if $p<0.05$. The percent of inhibition by an antinociceptive agent was determined for the acetic-acid-induced writhing and formalin tests using the following formula [26]:

Inhibition%=100(Control-Experiment)/Control

Results and Discussion

Analysis of the essential oil

The essential oil of the fresh leaves of *C. restingae* (OECR) was pale yellow in color and had an average yield of 0.83%. This yield compares favorably with that typically produced in this species, which is often much less than 1%. Only five compounds were identified, corresponding to 98.50% of the total oil contents (Table 1). Out of these, four were identified through the comparison of retention indices and mass spectra from the literature [22], while the structure of the main constituent (81.03%), named calyptrantone (1), was elucidated through NMR, UV and IR analysis.

In this study, the essential oil of a *C. restingae* proved to be very interesting due to the fact that it crystallized in the Clevenger tap, allowing it to be isolated in crystal form. Except for the major constituent, the other components identified were sesquiterpenes – (*E*)-caryophyllene (2.40%), *α*-humulene (1.52%), *β*-selinene (8.54%), and *α*-selinene (5.01%).

Spectral analyses of the calyptrantone (1): The UV spectrum revealed the presence of two bands with maximum absorption at 273 and 243 nm, consistent with the structure of leptospermona-type β-triketones [27]. The IR spectrum showed intense bands of carbonyl at 1730 and 1678 cm^{-1}, which correspond to the carbonyl stretching of ketones. The mass spectrum showed a molecular ion ($M^{+\bullet}$) m/z 224 compatible with the molecular formula $C_{12}H_{16}O_4$ and key fragments from the molecular ion *m/z* 43 (100%) for an acetyl portion. The molecular formula of 1, $C_{12}H_{16}O_4$, was determined on the basis of high-resolution ESI mass spectroscopy in the negative mode ($[M - H]^-$) at *m/z* 223.0915 ($C_{12}H_{16}O_4$, calc. 224). However, the structural elucidation of 1 was possible only after detailed analysis of the 1D and 2D NMR spectra data (Table 2).

The ^1H NMR spectra ($CDCl_3$) of compound 1 indicated that enoltautomers predominated inasmuch as an OH signal was present at a chemical shift of around 18.23 ppm, indicative of a very strong intramolecular hydrogen bond. These data indicated that it was in the keto-enol equilibrium (Figure 1), which was confirmed by a ^1H NMR study, carried out by adding drops of deuterated water (Figure 2). This experiment revealed a clear signal at 4.8 ppm, characteristic of hydrogen attached to oxygen, confirming the exchange of the enolic hydrogen for deuterium.

Heteronuclear correlations observed in the gHMBC spectrum allowed us to assign ring methyl signals. One signal was seen for the two methyl at C-2 and one signal for the two methyl at C-6. These correlations were also important to show the enolic hydrogen correlations (18.26 ppm) with carbon C-4 (109.4), C-5 (199.2), C-6 (52.0), C-7 (201.7) and C-8 (27.4) (Figure 3).

Finally, the proposed structure for calyptrantone (1) is supported by the similar structure of *β*-triketone leptospermone, isolated from *Leptospermum scoparium* [27]. Besides *Leptospermum*, to our knowledge, *β*-triketones are found in several genera of Myrtaceae such as *Backhousia*, *Baeckea*, *Callistemon*, *Calythrix*, *Campomanesia*, *Corymbia*, *Darwinia*, *Eucalyptus*, *Kunzea*, *Melaleuka* and *Xanthostemon*. It is interesting to note that *β*-triketones of the type 1, with methyl substituents in a ring of six members and an acyl side chain, are rare in natural products and this is the first time that *β*-triketone has been identified in the essential oil of a *Calyptranthes* species and also that calyptrantone has been recorded as a natural product.

Pharmacological activity

Writhing test: In the writhing tests, the 50 and 100 mg/kg doses of the EOCR reduced significantly ($p<0.001$) the number of writhing

RI[b] (calc.)	RI[c] (lit.)	Compounds	Peak area (%)			
			S1	S2	S3	Mean ± SD
1410	1417	(*E*)-caryophyllene	2.08	2.20	2.93	2.40 ± 0.46
1423	-	calyptrantone	82.83	83.42	76.85	81.03 ± 3.63
1447	1452	*α*-humulene	1.38	1.32	1.86	1.52 ± 0.30
1480	1489	*β*-selinene	7.38	7.77	10.46	8.54 ± 1.68
1487	1498	*α*-selinene	4.31	4.52	6.20	5.01 ± 1.04
TOTAL			**97.98**	**99.23**	**98.30**	

Table 1: Chemical composition of the essential oil from the fresh leaves of *Calyptranthes restingae* Sobral. [a]S1, S2 and S3, samples 1-3; SD, standard deviation; [b]RI (calc.), retention index on DB-5 column; [c]RI (lit.), retention index according to reference [22].

C	Chemical shifts-δ Calyptrantone		Observed correlation	Chemical shifts-δ Leptospermone [23]
	^{13}C	^1H –*J* (Hz)	HMBC	^{13}C
1	210.1			210
2	56.8			56.9
3	196.8			196.9
4	109.4			109.5
5	199.2	18.26, 1H, s	C-4; C-5; C-6; C-7; C-8	199.5
6	52			52.4
7	201.7	18.26, 1H, s	C-4; C-5; C-6; C-7; C-8	203.6
8	27.4	2.61, 3H, s	C-4; C-7	47.2
9 and 10	24.4	1.46, 6H, s	C-1; C-5; C-6; C-9 and C-10	24.3
11 and 12	23.9	1.37, 6H, s	C-1; C-2; C-3; C-11 and C-12	23.9

Table 2: NMR data for calyptrantone, the major constituent of the essential oil from the fresh leaves of *Calyptranthes restingae*.

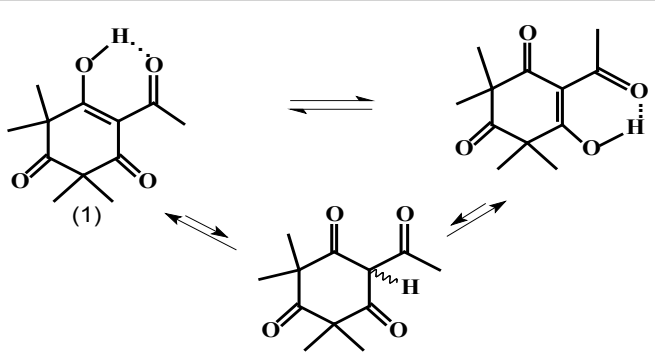
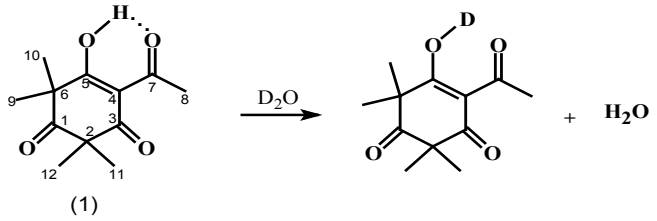


Figure 1: Calyptrantrone tautomers.



(1)

Figure 2: Experiment carried with the addition of drops of deuterated water.

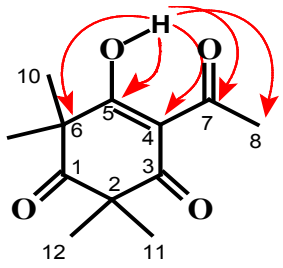


Figure 3: Fundamental heteronuclear correlations of C-H (gHMBC) in the determination of the methyl substituent position.

movements induced by the administration of the acetic acid solution (Table 3), producing an effect similar to that of morphine (3 mg/kg). However, naloxone (1.5 mg/kg, i.p.) antagonized the antinociceptive response of morphine from 9.8 ± 2.7 writhes in the acetic-acid-induced test, the reversal of the effect of EOCR at a higher dose (15.0 ± 2.0).

Acetic-acid-induced writhing is a standard, simple and sensitive test for measuring analgesia induced by both opioids and peripherally acting analgesics [28,29]. In this test, the pain is elicited by the injection of an irritant into the peritoneal cavity, which produces episodes of characteristic stretching (writhing) movements, making the inhibition of the number of episodes by analgesics easily quantifiable. These results also suggest that EOCR participates in the inhibition of the synthesis of prostaglandin, as the nociceptive mechanism involves the processing or release of arachidonic acid metabolites via cyclooxygenase (COX) and prostaglandin biosynthesis during abdominal writhing induced by acetic acid [24]. Furthermore, is possible to suggest the participation of the opioid system in the modulation of pain by this oil, whereas naloxone (1.5 mg/kg, i.p.) antagonized the antinociceptive effect of EOCR.

Formalin test: The Table 4 shows that EOCR inhibited the two phases of the formalin response, at the higher dose. In the first phase, EOCR (100 mg/kg) reduced licking time significantly ($p<0.001$) compared with the control group. In the second phase of the formalin test, the 50 and 100 mg/kg doses reduced pain significantly ($p<0.05$ and $p<0.001$, respectively).

Formalin test is a model of nociceptive response in two distinct phases involving different mechanisms. The first phase (neurogenic pain) results from the direct chemical stimulation of nociceptive afferent fibers, mainly C fibers, which can be suppressed by opioid analgesic drugs like morphine [30]. The second phase (inflammatory pain) results from the release of inflammatory mediators in the peripheral tissues and functional changes in the neurons of the spinal dorsal horn that, over the long term, promote facilitation of synaptic transmission at the spinal level [31], which is sensitive to the action of the majority of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, indomethacin and naproxen [32].

Rota-rod test: In the rota-rod test, mice treated with EOCR did not exhibit any significant alterations of motor performance at doses of 25, 50 or 100 mg/kg (Table 5).

The absence of alterations of motor performance in the rota-rod test indicates that the treatment with the oil did not affect the results of the previous tests in terms of alterations in motor performance.

Inflammation test: The Figure 4 shows the inhibitory effect of EOCR on carrageenan-induced response ($p<0.01$). The results obtained with the control group support the effect of EOCR since the vehicle presented no activity, and the control drug dexamethasone inhibited ($p<0.01$) the carrageenan-induced leukocyte migration to the peritoneal cavity.

The inflammation induced by carrageenan involves cell migration, plasma exsudation and the production of mediators, such as nitric oxide, prostaglandin E2, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, which are able to recruit leukocytes, such as neutrophils, in several experimental models [33]. EOCR inhibited the leukocyte migration induced by carrageenan and a putative mechanism associated with this activity may be the inhibition of the synthesis of many inflammatory mediators involved in cell migration. Furthermore, previous studies have shown that some terpenoid oil

Treatment	Dose (mg/kg)	Number of writhings[a]	% Inhibition
Vehicle	-	16.0 ± 1.2	-
EOCR	25	6.5 ± 2.5	59.4[c]
EOCR	50	2.3 ± 1.1[b]	85.6[d]
EOCR	100	0.8 ± 0.5[b]	95.0[d]
EOCR+NAL	100+1.5	15.0 ± 2.0	6.3
Morphine	3	0.4 ± 0.2[b]	97.5[d]
Morphine+NAL	3+1.5	9.8 ± 2.7	38.8

Table 3: Effect of EOCR or morphine on writhing induced by acetic acid. *n*=10 (per group). [a]Values represent mean ± S.E.M; [b]$p<0.001$ (one-way ANOVA and Dunnett`s test), significantly different from control; [c]$p<0.01$; [d]$p<0.001$ (Fisher`s test), significantly different from control.

Treatment	Dose (mg/kg)	Number of licks (s)			
		0-5 min		15-30 min	
		Score of pain[a]	% inhibition	Score of pain[a]	% inhibition
Vehicle	-	56.3 ± 6.2	-	31.8 ± 5.7	-
EOCR	25	51.8 ± 5.4	8.0	25.3 ± 6.0	20.4
EOCR	50	37.1 ± 8.9	34.1	9.8 ± 6.7[b]	69.2[d]
EOCR	100	22.0 ± 6.5[c]	60.9[d]	3.7 ± 3.2[c]	88.4[e]
Aspirin	200	22.4 ± 9.1[c]	66.0[e]	2.0 ± 1.3[c]	93.7[e]

Table 4: Effect of EOCR or aspirin on formalin-induced pain. *n*=10 (Per group). [a]Values represent mean ± S.E.M; [b]$p<0.05$; [c]$p<0.001$ (one-way ANOVA and Dunnett`s test), significantly different from control; [d]$p<0.05$; [e]$p<0.001$ (Fisher`s test), significantly different from control.

Treatment	Dose (mg/kg)	Time (s)[a]
Vehicle	-	180 ± 0.0
EOCR	25	180 ± 0.0
EOCR	50	180 ± 0.0
EOCR	100	171.8 ± 8.2
Diazepam	1.5	6.5 ± 3.5[b]

Table 5: Effect of EOCR or diazepam on the rota-rod test in mice. *n*=10 (Per group). [a]Values represent mean ± S.E.M; [b]$p<0.001$ (one-way ANOVA and Dunnett`s test), significantly different from control.

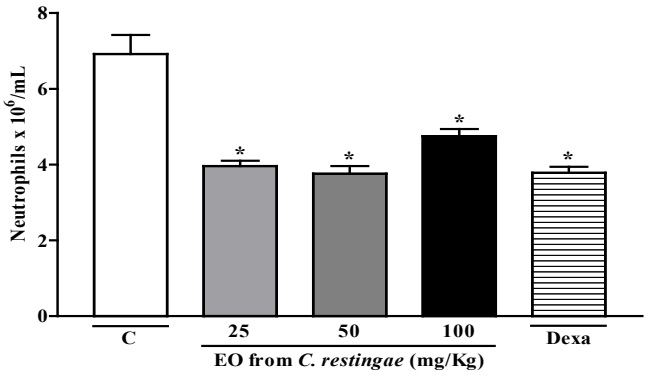


Figure 4: Effect of the EOCR on leukocyte migration into the peritoneal cavity induced by carrageenan. Each value represents the mean ± S.E.M. *p<0.01 (one-way ANOVA and Dunnett's test), significantly different from control.

constituents possess antinociceptive and anesthetic activities in animal experiments [34,35]. The antinociceptive effect of the essential oil of aromatic plants such as *Hyptis pectinata*, which has (E)-caryophyllene as a major constituent (45.1%), is reversed completely by the opioid agonist naxolone [36]. Moreover, Fernandes et al. propose that (−)-(*E*)-caryophyllene is able to markedly reduce the formation of edemas induced by bradykinin and PAF in mice [37]. In this study, (−)-(*E*)-caryophyllene was effective in diminishing the production of TNFα. All these findings suggest that calyptrantone (81.03%), β-selinene (8.54%), α-selinene (5.01%), (*E*)-caryophyllene (2.40%) and α-humulene (1.52%), derived from the essential oil of *C. restingae*, might represent important tools for the management and/or treatment of pain and inflammatory processes.

Conclusions

Therefore, it is possible to conclude that this essential oil possesses antinociceptive and anti-inflammatory properties, probably via opioid receptors or mediated by the inhibition of the synthesis of inflammatory mediators, such as prostaglandin. Further studies currently in progress will enable us to understand the precise mechanisms.

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