A New \( \beta \)-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%), \( \alpha \)-humulene (1.52%), \( \beta \)-selinene (8.54%), \( \alpha \)-selinene (5.01%). The structure of the major compound, calyptrantone, was elucidated by \( \mathrm{H} \) and \( \mathrm{C} \)-NMR. This is the first time that a \( \beta \)-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*; \( \beta \)-triketone; Calyptrantrone; 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 essential oils of *Eugenia uniflora* [11] and *E. candolleiana* 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 \( \beta \)-triketone, named calyptrantrone 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: \( 8.47.325^\circ/36.58.414^\circ \), 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 bottles.

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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 W Scientific DB-5MS (Folsom, CA, USA) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness). Helium (99.9999%) 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 x 0.25 mm i.d. x 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 C1-C4 linear hydrocarbon mixture and calculated according to Van den Doel and Kratz equation. The volatile components were analyzed by means of GC-MS 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 calyptrantrone 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 H-1 and 13C-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 (CDCl3) was used as the solvent and tetramethylsilane (TMS) as the internal standard.

**Spectral data of calyptrantrone**

Calyptrantrone (1) [4-acetyl-5-hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione] was obtained as a pale yellow oil; UV (MeOH) λmax 273 e 243 nm; IR (KBr) νmax 3757, 3679, 3653, 3443, 3328, 2945, 2866, 2717, 2639, 2499, 2369, 2333, 1730, 1678, 1564, 1477, 1423, 1364, 1350, 1215, 1171, 1049, 961, 938, 874, 839, 775 e 646 cm⁻¹;ė HSRESIMS m/z 223.0915 [M-H]-, EIMS m/z 224[M⁺]¹ (100%), 70 and 154; and secondary m/z 55, 96, 112, 129, 137, 161, 181, 181, 196, 209, 194-MNR (400.21 MHz, CDC13/TMS): δ1 18.26 (1H, s, H3-18); δ2 2.61 (3H, s, H-8); 1.46 (6H, s, H-9 and H-10); 1.37 (6H, s, H-6); 1.30 (6H, s, H-11 and H-12); 13C-NMR (100.6 MHz, CDC13/TMS): δC 210.1 (C-1); 201.7 (C-7); 199.2 (C-5); 196.8 (C-3); 190.4 (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/UFSN 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. EOCCR 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 mice 0.5 h before the acetic acid injection. An additional group was pretreated with 1.5 mg/kg of naxalone (i.p.), a nonselective opioid antagonist, 15 min before the i.p. administration of the vehicle (control), EOCCR (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, EOCCR (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° 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, EOCCR (25, 50, and 100 mg/kg, i.p.), or diazepam (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 EOCCR (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
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 (CDCl₃) of compound 1 indicated that enolatromers predominated inasmuch as an OH signal was present 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 for deuterium.

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, Calytrix, Campomanesia, Corymbia, Darwinia, Eucalyptus, Kunzea, Melaleuca 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 EOOCR reduced significantly (p<0.001) the number of writhing episodes. 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]:

\[
\text{Inhibition} \% = \frac{100 \times (\text{Control} - \text{Experiment})}{\text{Control}}
\]

### Table 1: Chemical composition of the essential oil from the fresh leaves of *Calyptranthes restingae* Sobral.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Peak area (%)</th>
<th>RIb (calc.)</th>
<th>RIc (lit.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1410 (E)-caryophyllene</td>
<td>2.08</td>
<td>2.20</td>
<td>2.93</td>
</tr>
<tr>
<td>calyptrantone</td>
<td>82.83</td>
<td>83.42</td>
<td>76.85</td>
</tr>
<tr>
<td>α-humulene</td>
<td>1.38</td>
<td>1.32</td>
<td>1.86</td>
</tr>
<tr>
<td>β-selinene</td>
<td>7.38</td>
<td>7.77</td>
<td>10.46</td>
</tr>
<tr>
<td>α-selinene</td>
<td>4.31</td>
<td>4.52</td>
<td>6.20</td>
</tr>
<tr>
<td>TOTAL</td>
<td>97.98</td>
<td>99.23</td>
<td>98.30</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>C</th>
<th>Chemical shifts-δ</th>
<th>Observed correlation</th>
<th>Chemical shifts-δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H – J (Hz)</td>
<td>HMBC</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>210.1</td>
<td></td>
<td>210</td>
</tr>
<tr>
<td>3</td>
<td>196.8</td>
<td></td>
<td>196.9</td>
</tr>
<tr>
<td>4</td>
<td>109.4</td>
<td></td>
<td>109.5</td>
</tr>
<tr>
<td>5</td>
<td>199.2</td>
<td>18.26, 1H, s</td>
<td>199.5</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td></td>
<td>52.4</td>
</tr>
<tr>
<td>7</td>
<td>201.7</td>
<td>18.26, 1H, s</td>
<td>203.6</td>
</tr>
<tr>
<td>8</td>
<td>27.4</td>
<td>2.61, 3H, s</td>
<td>47.2</td>
</tr>
<tr>
<td>9 and 10</td>
<td>24.4</td>
<td>1.46, 6H, s</td>
<td>24.3</td>
</tr>
<tr>
<td>11 and 12</td>
<td>23.9</td>
<td>1.37, 6H, s</td>
<td>23.9</td>
</tr>
</tbody>
</table>

**Figure 1: Calyptranthes tautomers.**

**Figure 2: Calyptrantone tautomers.**
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 exudation 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Number of writhing†</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>60.0 ± 6.2</td>
<td>-</td>
</tr>
<tr>
<td>EOCR</td>
<td>25</td>
<td>0.4 ± 0.2</td>
<td>97.5d</td>
</tr>
<tr>
<td>EOCR</td>
<td>50</td>
<td>2.3 ± 1.1b</td>
<td>85.6e</td>
</tr>
<tr>
<td>EOCR</td>
<td>100</td>
<td>0.8 ± 0.5b</td>
<td>95.0f</td>
</tr>
<tr>
<td>EOCR+NAL</td>
<td>100+1.5</td>
<td>15.0 ± 2.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Morphine</td>
<td>3</td>
<td>0.4 ± 0.2</td>
<td>97.5f</td>
</tr>
<tr>
<td>Morphine+NAL</td>
<td>3+1.5</td>
<td>9.8 ± 2.7</td>
<td>38.8</td>
</tr>
</tbody>
</table>

Table 3: Effect of EOCR or morphine on writhing induced by acetic acid. n=10 (Per group). Values represent mean ± S.E.M; †p<0.001 (Fisher’s test), significantly different from control; £p<0.01; ¥p<0.001 (one-way ANOVA and Dunnett’s test), significantly different from control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Number of licks (s)</th>
<th>0-5 min</th>
<th>15-30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>56.3 ± 6.2</td>
<td>31.8 ± 5.7</td>
<td>-</td>
</tr>
<tr>
<td>EOCR</td>
<td>25</td>
<td>51.8 ± 5.4</td>
<td>0.0</td>
<td>20.4</td>
</tr>
<tr>
<td>EOCR</td>
<td>50</td>
<td>37.1 ± 8.9</td>
<td>34.1</td>
<td>69.2£</td>
</tr>
<tr>
<td>EOCR</td>
<td>100</td>
<td>22.0 ± 6.5†</td>
<td>60.9£</td>
<td>88.4f</td>
</tr>
<tr>
<td>Aspirin</td>
<td>200</td>
<td>22.4 ± 9.1†</td>
<td>66.0£</td>
<td>93.7£</td>
</tr>
</tbody>
</table>

Table 4: Effect of EOCR or aspirin on formalin-induced pain. n=10 (Per group). *Values represent mean ± S.E.M; †p<0.01; ¥p<0.001 (one-way ANOVA and Dunnett’s test), significantly different from control; £p<0.05; ¥p<0.001 (Fisher’s test), significantly different from control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Time (s)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>180 ± 0.0</td>
</tr>
<tr>
<td>EOCR</td>
<td>25</td>
<td>180 ± 0.0</td>
</tr>
<tr>
<td>EOCR</td>
<td>50</td>
<td>180 ± 0.0</td>
</tr>
<tr>
<td>EOCR</td>
<td>100</td>
<td>171.8 ± 8.2</td>
</tr>
<tr>
<td>Diazepam</td>
<td>1.5</td>
<td>6.5 ± 3.5†</td>
</tr>
</tbody>
</table>

Table 5: Effect of EOCR or diazepam on the rota-rod test in mice. n=10 (Per group). *Values represent mean ± S.E.M; †p<0.001 (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 effective in diminishing the production of TNFα. All these findings suggest that calyptranone (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 processes 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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References


