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

**Keywords:** Myrtaceae; *Calyptranthes restingae*; β-triketone; Calyptrantron; 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 anti-hypertension 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 "murtu", 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 calyptrantron 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 bottles and stored in a dark place at 4 °C.

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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 × 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 C1-C, linear hydrocarbon mixture and calculated according to Van den Dool 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 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 1H-, and IR 1000 spectrometer. Melting points were measured in a Toledo FP90 digital Mettler apparatus and are uncorrected. The 1H-, 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 1H-, and 13C-NMR spectra and 2D experiments were obtained on a Bruker system and other published mass spectra [22].

Spectral data of calyptrantone

Calyptrantone (1) [4-acetyl-5-hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-diene] was obtained as a pale yellow oil; UV (MeOH) λmax 273 e 243 nm; IR (KBr) νmax 3757, 3767, 3653, 3617, 3443, 3328, 3319, 3198, 3075, 3066, 2945, 2866, 2717, 2639, 2499, 2369, 2333, 1730, 1678, 1564, 1477, 1423, 1364, 1350, 1215, 1171, 1049, 916, 938, 874, 839, 789, 725 e 664 cm−1; HRESIMS m/z 232.0915 [M-H]-, EIMS m/z 224[M+H]+ (100%), 70 and 154; and secondary m/z 55, 96, 111, 126, 139, 157, 161, 181, 196, 209; 1H-NMR (400.21 MHz, CDCl3/TMS): δH 18.26 (1H, s, Hkeletal); 2.61 (3H, s, H-8); 1.46 (6H, s, H-9 and H-10); 1.37 (6H, H-1 and H-10); 1.37 (6H, H-11 and H-12); 13C-NMR (100.16 MHz, CDCl3/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/UFS N° 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 the mice 0.5 h before the acetic acid injection. An additional group was pretreated with 1.5 mg/kg of naltrexone (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 intense 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.), 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 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...
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]:

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

**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 leptospermone-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+•) m/z 224 compatible with the molecular formula C12H16O4, and key fragments from the molecular ion m/z 143 (100%) for an acetyl portion. The UV spectrum was consistent with the structure of leptospermona-type β-triketones of the type 1, with methyl substituents in a ring of six members and an acyl side chain, 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 for *C. restingae*. Finally, the proposed structure for calyptrantone (1) is supported by the similar structure of β-triketone leptospernone, isolated from *Leptospermum scoparium* [27]. Besides *Leptospermum*, to our knowledge, β-triketones are found in several genera of Myrtaceae such as *Backhousia*, *Baeckea*, *Callistemon*, *Calycithrix*, *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.

| Compounds      | Observed correlation | Chemical shifts-δ HMBC  | Chemical shifts-δ Leptospernone [23] | Peak area (%) |
|----------------|----------------------|-------------------------|-------------------------------------|--------------|
|                | 13C                  | 1H – J (Hz)             |                                     |              |
| 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                 | 26.1, 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 1:** Chemical composition of the essential oil from the fresh leaves of *Calyptranthes restingae* Sobral. *S1, S2 and S3, samples 1-3; SD, standard deviation; *RI* (calc.), retention index on DB-5 column; *RI* (lit.), retention index according to reference [22].

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

![Figure 1: Calyptrantrone tautomers.](image-url)
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 treatments exhibit any significant alterations of motor performance at doses of 25, 50 or 100 mg/kg (Table 5).

**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.

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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 naltrexone [36]. Moreover, Fernandes et al. propose that (−)-(E)-caryophyllene is effective in diminishing the production of TNFα. All these findings suggest that caryophyllane (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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