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Antinociceptive effect of neo-clerodane diterpenes obtained from *Baccharis flabellata*

Matías Funes[a], María F. Garro[b,c], Rodrigo D. Tosso[b], Alejandra O. Maria[b], José R. Saad[b], Ricardo D. Enriz[b,c,*]

[a] INTEQUI-CONICET, Universidad Nacional de San Luis, Área de Química Orgánica, Almirante Brown 1455, D5700HGC San Luis, Argentina
[b] Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco 915, 5700 San Luis, Argentina
[c] Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-CONICET), Chacabuco 915, 5700 San Luis, Argentina

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ABSTRACT

We report here for the first time antinociceptive effects of extracts from *Baccharis flabellata*. Two extracts in this analysis, one obtained in summer and the other during winter time. Our results indicate that both extract show strong antinociceptive effects, being the extracts obtained during the summer significantly more active.

Our results suggest that this activity is mainly due to the presence of the diene-acid clerodane ent-15,16-epoxy-19-hydroxy-1,3,13(16),14-clerodatetraen-18-oic acid (DAC) and its dimer called DACD. Employing naloxone as an antagonist of opioid receptors, we demonstrated that both compounds act on opioid receptors, being the antinociceptive effect of DACD stronger than DAC. Thus, the antinociceptive activity of DACD was almost two times stronger than DAC (44.8 over 24.6 s in the hot-plate test) after one hour of treatments.

In order to better understand the mechanism of action at molecular level of these compounds, we conducted a molecular modeling study analyzing the molecular interactions of DAC and DACD complexes with the κ-ORs. Our results suggest interactions for both DAC and DACD with Gln115, Val118, Tyr119, Asn122 and Tyr313 stabilizing their complexes; however, these interactions are significantly stronger for DACD with respect to DAC. This finding could explain why DACD have a higher affinity for the κ-ORs. These results are in agreement with the obtained antinociceptive effect. In addition, our results indicate that these neoclerodanes would have a mechanism of action similar to that of salvinorin A; such information can be very useful for the design of new inhibitors of κ-ORs.

1. Introduction

The main three families of plants with medicinal use in Argentina are *Asteraceae*, *Fabaceae*, and *Solanaceae*. Members of these families have been used for the prevention and relief of medical disorders since ancient time, therefore their metabolites have been extremely studied and an enormous amount of chemical information is now available [1–3]. In Argentina, *Asteraceae* comprises ca. 1490 species (indigenous and introduced) [4], and 272 native taxa (ca. 18%) have been reported with medicinal uses [5]. In particular, the genus *Baccharis* is one of the most important considering its enormous relevance regarding its medicinal, commercial, and biological applications [6]. Thirty-six species of the ninety-six growing in Argentine [7] have medicinal properties, and the majority of them have been phytochemically studied. *Baccharis* is an exclusively American genre, with around 500 species; most of them live in tropical regions of the continent, although there are many species of temperate and temperate-cold zones, some of which grow in the so-called Pampa Argentina. Some species of the genus *Baccharis* are known for the effect produced by their chemical principles. Thus, some of them, generically called “carquejas”, have an intensive use in popular medicine. Numerous studies have shown that the “carquejas” have antioxidant, anti-inflammatory, analgesic, anti-

Abbreviations: AMSL, Above mean sea level; i.p., Administered via the intraperitoneal route; ANMAT, Administración Nacional de Medicamentos, Alimentos y Tecnología Médica; CNS, Central nervous system; CTLC, Centrifugal thin layer chromatography; DAC, ent-15,16-epoxy-19-hydroxy-1,3,13(16),14-clerodatetraen-18-oic acid; DACD, (1R,4S,4aS,4bR,5S,6R,8aS,10aR)-3-(2-((1S,2R,4aS,8aR)-5-carboxy-4a-(hydroxymethyl)-1,2-dimethyl-1,2,3,4,4a,8a-hexahydronaphthalen-1-yl) ethyl)-5-(2-(furan-3-yl)ethyl)-8a-(hydroxymethyl)-5,6-dimethyl-1,4,4a,4b,5,6,7,8,8a,10a-decahydro-1,4-epoxyphenanthrene-9-carboxylic acid; MD, Molecular Dynamics; NO, Nitric oxide; SalA, Salvinorin A; *s.c.*, Subcutaneous; SAR, Structure-Activity Relationship; κ-ORs, κ-opioid receptors

* Corresponding author at: Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-CONICET), Universidad Nacional de San Luis, Chacabuco 915, 5700 San Luis, Argentina.
E-mail address: denriz@unsl.edu.ar (R.D. Enriz).

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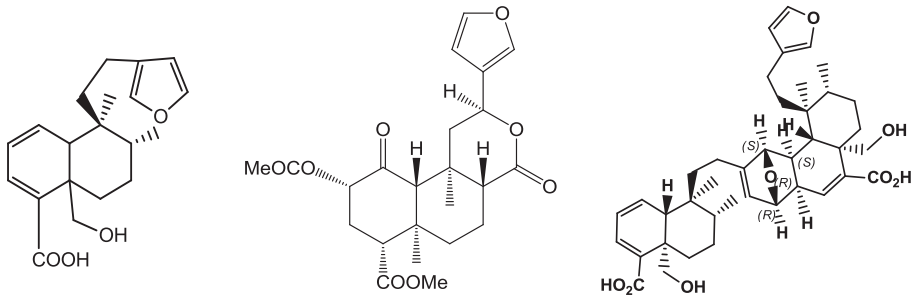

Fig. 1. Structures of **SalA, DAC** and its natural [4 + 2] photocycloaddition dimer **DACD**.

hepatotoxic and anti-mutagenic activities. Most of its uses in traditional medicine have been validated and these plants appear in the official pharmacopoeias of most of the countries of South America as specific plants for digestive aids. Gené et al. have reported that *B. trimera* shows strong anti-inflammatory and analgesic properties [8]. Very recently we have reported a dimer neoclerodane diterpene isolated from *B. flabellata* possibly formed by a [4 + 2] photo-cycloaddition of DAC (Fig. 1) called DACD [9]. Clerodane diterpenoids are a widespread class of secondary metabolites [10] which have been found in several plant species from various families as well as in organisms from other taxonomic groups such as fungi, bacteria, and marine sponges [11]. From a chemical point of view, clerodane diterpenes are bicyclic diterpenoids and their basic skeleton is divided into two fragments: a fused ring decalin moiety (C-1–C-10) and a six-carbon side chain at C-9. Clerodane diterpenoids constitute a large class of natural products and the number of these compounds has grown rapidly from several years ago. A comprehensive review on different aspects of clerodane diterpenoids was published by Li et al. [11]. Clerodane diterpenoids have attracted interest in recent years as a result of their noteworthy biological activities: antiparasitic [12], antifungal and antibacterial [13], antitumor [14] and anti-inflammatory [15]. In particular, much attention has been given to the antinociceptive activities found for SalA, non-nitrogenous opioid ligand of the κ-ORs [16,17].

Previous studies on the biological effects of essential oils obtained from *B. flabellata* have only reported antibacterial activities for *Staphylococcus aureus* [18]. However, neither analgesic nor antinociceptive activities have been reported to date for the extracts of any *Baccharis*. Having now the advantage of knowing about the presence of abundant amounts of DAC and DACD in the *B. flabellata* extracts, the question that arises is whether these extracts could have some type of antinociceptive activity. To answer this question, a study of the antinociceptive activity was made, taken the extracts at different times of the year, considering that the amount of DACD increases with the solar radiation. To complete this study, the activity of the supposed active principles DAC and DACD was also evaluated. In addition, we simulated the behavior of these clerodane diterpenoids over the k-ORs. From a molecular modeling study our results suggest that DAC and DACD act as agonists of the k-ORs. To better understand the effect at molecular level, combined techniques of docking and MD simulations have been used, which have allowed us to determinate the molecular interactions of these compounds at the molecular target. In this study we have also included SalA, which is a natural neoclerodane with potent opioid analgesic effect.

2. Material and methods

2.1. General experimental procedures

Solvents were analytical grade or were purified by standard procedures prior to use. Silica gel GF_{254} and Silica gel 60 (0.040–0.063 mm) were purchased at Merck (Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were performed on Bruker AC-200 spectrometer (200 MHz), 2D NMR spectra were measured as usual. We used $CDCl_3$ as solvent for ^{1}H and ^{13}C NMR spectra; chemical shifts were referenced to $CDCl_3$ residual signals, in the case of $CDCl_3$ at δ_H 7.26 and the central peak at δ_{13C} 77.0. EIMS: at 70 eV on GCQ Plus instrument. UV–Vis absorption spectra were taken with a Lambda 25 Perkin Elmer (Madrid, Spain) spectrometer.

2.2. Plant material and extraction procedure

Aerial parts (comprising leaves and stems, 100 g) of *B. flabellata* were collected during December of 2017 at 1270 m AMSL in Potrero de los Funes town, San Luis hills, Argentina (33° 11′ 90.88” S, 66° 15′ 42.63” W). All specimens were authenticated by Prof. Dr. Elisa Petenatti and each batch was deposited at the Herbarium of the Universidad Nacional de San Luis (L.A. Del Vitto & E.M. Petenatti # 9436). Leaves were extracted with methanol in portions of 200 ml each, immediately after being cut from the plant at room temperature.

2.3. Isolation of furan neoclerodanes

As it was previously reported [9], we employed here the same separative techniques. Thus, preparative TLC and Centrifugal TLC methods were employed to obtain DACD and DAC. Purity of isolated compounds was corroborated by HPLC-DAD, for details see Fig. S21 and S23 at Supplementary data.

2.4. Bioassays

2.4.1. Animals

The experiments were performed on Rockland mice of either sex (25–30 g) with free access to standard food and water, in a 12 h day–night cycle (lights on from 07:00 to 19:00 h), at a constant temperature of 22 ± 3 °C (with periodic cycles of air changes) and a relative humidity of about 50–60%. Acclimatization of animals was done for two days before the beginning of the experiment. The animals were randomly assigned to the different groups. All the animals were obtained from the Bioterium of the Facultad de Química, Bioquímica y Farmacia of Universidad Nacional de San Luis (Argentina) and the experiments were in compliance with the ANMAT No. 6344/96. [19] for animal care guidelines and were also authorized by Institutional Committee for the Care and Use of Laboratory Animals (Acronym: CICUA) of our institution (protocol No. F-206/15 in Resolution 324–16).

2.4.2. Antinociceptive activity

The hot-plate test was used to measure response latencies according to the method described by Eddy and Leimbach, with minor modifications [20]. Each mouse was placed on a hot plate kept at 56 ± 1 °C, after 30 min of animal's injections. The latency in seconds