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Isolation and Quantification of Pinitol, a Bioactive Cyclitol, in *Retama* spp.

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The genus *Retama* (Fabaceae) is widely distributed in the Mediterranean region. In the present study, pinitol (3-*O*-methyl-*chiro*-inositol), an anti-inflammatory and antidiabetic molecule, was isolated from aerial parts of *R. monosperma*, and its structure established on the basis of spectroscopic techniques (1D/2D NMR) and MS. Identification and quantification of pinitol in *R. raetam* and *R. sphaerocarpa* were also performed. *R. monosperma* had the highest concentration of pinitol (2.3%). The presence of pinitol in aqueous extracts of *Retama* spp. may explain the adaptation of these plants to drought and salinity. Furthermore, pinitol could be considered as a mediator in the anti-inflammatory and hypoglycemic activities of *Retama* spp., which are traditionally used to treat diabetes.

Keywords: *Retama*, Pinitol, Cyclitol, 3-*O*-Methyl-*chiro*-inositol.

Traditional medical knowledge has two potential values: one as an easily accessible and low-cost source of medicines for primary health care; and the other as the source of lead compounds for the development of new drugs. Many researchers are nowadays interested in giving scientific authentication to the activity of medicinal plants used in traditional medicine around the world and seeking explanations for their mechanisms of action [1].

One such group of plants currently used by the Mediterranean population in the treatment of different ailments, includes plants belonging to the genus *Retama* (Fabaceae). The aerial parts of *R. monosperma* (L.) Boiss., *R. raetam* Forskk. and *R. sphaerocarpa* Boiss. are traditionally used to prepare decoctions for the treatment of diabetes, hypertension, and rheumatism [2-3]. Moreover, numerous *in vitro* and *in vivo* studies have shown different pharmacological properties, including hypoglycemic, antihypertensive, anti-inflammatory, antibacterial, antifungal, diuretic and analgesic activities [4-8]. Phytochemical studies have shown that these species are very rich in flavonoids (isoflavones) [9-10] and alkaloids (quinolizidine and bipiperidyl) [11].

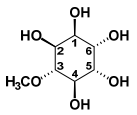

Figure 1: Structure of pinitol (3-*O*-methyl-*chiro*-inositol).

Continuing our research of bioactive compounds from the *Retama* genus, we have isolated a cyclitol named pinitol (3-*O*-methyl-*chiro*-inositol; Figure 1) from a butanolic extract of *R. monosperma*, by column chromatography on silica gel, and identified from ^{1}H, ^{13}C, COSY, NOESY, HSQC, and HMBC NMR spectroscopic, and mass spectral data.

In order to compare the quantity of pinitol in different *Retama* spp., aerial parts of *R. monosperma*, *R. raetam* and *R. sphaerocarpa* were extracted with water and the extracts lyophilized. Pinitol, after derivatization, was quantified in the three *Retama* species by gas chromatography–mass spectrometry (GC-MS); the results are expressed as percentage of dry plant weight (Table 1).

Table 1: Pinitol content of three species of *Retama*.

Species	Yield of aqueous extract (%)	% Pinitol in aqueous extract	% Pinitol in dry plant
R. monosperma	14.1	16.3	2.3
R. raetam	20.3	8.8	1.8
R. sphaerocarpa	25.5	7.4	1.9

R. monosperma showed the highest content (2.3%) of pinitol among the three species studied, and its aqueous extract was very rich in pinitol (16.3%). The major presence of pinitol in *R. monosperma* might be related to the fact that this plant material was collected from the "El Rompido" spit, which is a sand dune area with high salinity, especially from June to September. Pinitol, as well as other carbohydrates like sucrose and mannitol, has been observed in plants under osmotic stress [12]. Further research should be performed to confirm the role of pinitol as an osmoprotectant in *R. monosperma*.

R. monosperma, locally known as "retama blanca" (white bridal broom), is a legume shrub mainly established on Mediterranean coastal sand and dune areas [13]. We previously reported that *R. monosperma* aqueous extract exerted protective effects in an experimental murine model of Crohn´s disease [6]. The anti-inflammatory activity of *R. monosperma* was related to its content of flavonoids, but the isolation of pinitol from that aqueous extract suggests that this compound could also have a synergistic effect in the anti-inflammatory activity, since pinitol has been described as an inhibitor of the NF-κB inflammatory pathway [14].

Furthermore, pinitol has been reported to exert insulin-like effects and to play a positive role in insulin resistance through the PI3K/Akt signaling pathway in Type 2 diabetes mellitus [15]. Thus, it could be one of the active principles involved in the antidiabetic activity of *Retama* species, which are traditionally used to treat this ailment [4].

Experimental

General: Nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Bruker Avance 500 spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). Chemical shifts (δ, in ppm) were referenced to TMS, and *J* was expressed in Hz. EI-MS was taken on a Micromass Autospec (70eV) spectrometer.

Chemicals: Methanol, ethyl acetate, trichloromethane, and *n*-butanol (*n*-BuOH) were obtained from Panreac® (Barcelona, Spain), and pyridine from AppliChem® (Barcelona, Spain). Derivatization reagents BSTFA (*N,O*-bis(trimethylsilyl)acetamide) and TMCS (trimethylchlorosilane) were purchased from Supelco® (Madrid, Spain). All chemicals were of analytical reagent grade.

Plant material: Aerial parts of *R. monospema* were collected in the "El Rompido" spit (Huelva, SW Spain) in September 2010; those of *R. raetam* in Lota, Souk El Ténine (Béjaïa, Algeria) in April 2011; and *R. sphaerocarpa* in Bousselam, Bouandas (Setif, Algeria), in March 2011. The plant species were identified by one of our research team (JBGF), and a voucher specimen of each species was deposited in the herbarium of the University of Seville.

Extraction, isolation and identification* of *pinitol: The dried aerial parts of *R. monosperma* (395 g) were ground to a fine powder. The powder was successively extracted in a Soxhlet extractor with *n*-hexane for 24 h, and with methanol (MeOH) for 48 h. The methanolic extract was evaporated to dryness (90.3 g), suspended in 50 mL of distilled water, and then extracted successively with trichloromethane ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield 3 fractions: $CHCl_3$ (4.3 g), EtOAc (3.0 g), and *n*-BuOH (6.2 g).

A portion of the *n*-BuOH extract (2.5 g) was submitted to Silicagel column chromatography, eluted with a mixture of EtOAc-MeOH-H_2O (80:0.5:0.5; 80:1:1; 80:3:3). A total of 284 fractions of 25 mL were collected and monitored by thin-layer chromatography (TLC). Fractions from 95 to 135 were mixed and compound **1** (110 mg, 0.07% yield) was obtained as white needles after recrystallization with acetone. Compound **1** was identified as pinitol by comparison of its spectral features with available literature data [16].

Pinitol quantification by GC/MS: Air-dried aerial parts of *R. monosperma*, *R. raetam* and *R. sphaerocarpa* were ground to fine powders. One hundred g of powder was extracted in water with agitation for 1 h at 70ºC. The resulting extracts were then filtered and lyophyllized. Solutions of the three *Retama* spp. extracts (2.5 mg/ mL) and stock solutions of standard pinitol (Sigma®) at 1, 5, 10, 25, 50, and 100 µg/mL were prepared in distilled water. Fifty µL of each of these solutions was transferred to 250 µL mini-vials and freeze-dried to obtain anhydrous conditions prior to derivatization. Standards of pinitol and samples were dissolved in a mixture of pyridine/BSTFA/TMCS (10:5:1, v/v/v, 100 µL). Sylilated derivatives were injected on an Agilent 6890N gas chromatograph (Waldbronn, Germany), equipped with a polydimethylsiloxane DB-5MS capillary column (30 m x 250 µm) (Supelco®, Bellefonte, PA, USA) and a mass detector Autospec-Q.

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