Fitoterapia 113 (2016) 139–143

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Poligalen, a new coumarin from *Polygala boliviensis*, reduces the release of TNF and IL-6 independent of NF-kB downregulation

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ARTICLE INFO

Article history:
Received 28 May 2016
Received in revised form 18 July 2016
Accepted 31 July 2016
Available online 2 August 2016

Keywords:
Polygala boliviensis
Polygalaceae
Cytokines
Coumarin immune modulation

ABSTRACT

An unusually substituted coumarin, named poligalen, was isolated from a chloroform extract of the aerial parts of *Polygala boliviensis*. This coumarin was identified by one- and two-dimensional NMR techniques, and the structure of the compound was confirmed by X-ray diffraction. Poligalen exhibits immunomodulatory effects, reducing the levels of IL-6 and TNF after LPS stimulation in peritoneal macrophages. However, poligalen potentiates NF-kB activation.

1. Introduction

Polygala boliviensis A. W. Benn is a species of Polygalaceae, a family that includes approximately 1300 species distributed over 19 genera [1]. The *Polygala* L. genus contains approximately 725 species, the most species of any genus in the Polygalaceae family. Of these 725 species, 400 species are neotropical and can be found in open areas [2]. Marques and Pastore [3] found that the Polygalaceae family accounts for 130 species in Brazil, with *Polygala* being the most representative genus of the Polygalaceae family. Species are distributed throughout Brazilian "cerrado" and rupestrian fields.

Species of the Polygalaceae family are known to contain various classes of secondary metabolites that exhibit significant biological activity [4]. Several different compounds, including triterpene saponins [5,6], xanthones [7], coumarins, flavonoids [8,9] and lignans [4] have been isolated from species of *Polygala* genus. One specific characteristic of species within the Polygalaceae family is the presence of methyl salicylate, which is found mainly on the roots of the plants [10].

Polygala species are commonly used in folk medicine as topical anesthetics [11]. Analgesic effects may be related to the anti-inflammatory action of these plants. Studies have demonstrated the anti-inflammatory effect of species such as *P. tricornis*, which contains compounds with anti-neuroinflammatory activity [6]. Studies have also described the anti-inflammatory effects of *P. tenuifolia*, which is used to treat inflammatory diseases such as asthma and bronchitis [12], and *P. fruticose*, which is used in the treatment of intestinal inflammations [13].

During inflammation, the host responds with a series of immune reactions to neutralize invading pathogens, repair injured tissues, and promote wound healing. These reactions are mainly triggered by the release of pro-inflammatory cytokines [14]. Chemokines are cytokines with central roles in leukocyte physiology, chemotaxis, the control of basal traffic, and inflammatory responses. Chemokines include interleukins (IL), interferons (IFN), colony stimulating factors, growth factors and tumor necrosis factors (TNF) [15].

The anti-inflammatory effects that some species of *Polygala* exhibit have been attributed to immunomodulatory effects: species, such as *P. paniculata* [16], *P. tenuifolia* [17] and *P. molluginifolia* [18], effectively regulate pro-inflammatory cytokine levels.

This paper describes the isolation and identification of a new coumarin named poligalen from *P. boliviensis*. This paper also evaluates the action of poligalen in the regulation of pro-inflammatory cytokines.

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http://dx.doi.org/10.1016/j.fitote.2016.07.021
0367-326X/

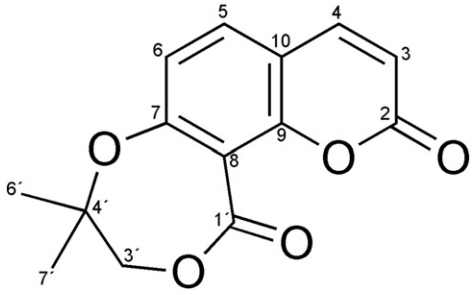

Fig. 1. Chemical structure of poligalen.

2. Experimental

2.1. General experimental procedures

Electrospray mass spectrometry (ESI-MS) analyses were conducted on a Shimadzu chromatographic system (LCMS-2310) and the HRESIMS in a Bruker MicroTof equipment. Nuclear Magnetic Resonance (NMR) spectroscopy (one and two-dimensional) was performed on a Varian INOVA 500 spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). Pyridine-d_5 was used as the solvent, with tetramethylsilane (TMS) as internal standard. The UV spectrum was recorded in acetonitrile on Varian Prostar 330 PDA detector. Conventional chromatographic methods were used for column chromatography (CC) [silica gel 60 (Acros, 0.063–0.200 mm)]. Thin-layer chromatography (TLC) silica gel plates (Merck) revealed with iodine and/or under ultraviolet (UV) light (254/366 nm) were used to monitor the chromatographic purification procedures. Crystals suitable for X-ray structure analysis were obtained by slow evaporation in chloroform ($CHCl_3$) at room temperature.

2.2. Plant material

Plant material was collected on July 2013 in Feira de Santana, Bahia. The material was identified by Prof. José Floriano Barea Pastore by comparing the collected material with a voucher specimen (HUEFS 168956) deposited at the Herbarium of the Universidade Estadual de Feira de Santana (HUEFS).

2.3. Extraction and isolation

Dried and powdered *P. boliviensis* aerial parts and roots (8.16 kg) were extracted three times with methanol at room temperature for 48 h and evaporated under reduced pressure. Then, the extract (1.66 kg – corresponding at 20.34% of dry material) was suspended in water and successively partitioned between hexane, $CHCl_3$ and ethyl acetate (EtOAc). The chloroform portion (291.11 g – 17.54% of methanolic extract) was subjected to column chromatography over silica gel 60 (0.063–0.200 mm) and eluted successively using a gradient of Hexane–EtOAc mixtures with increasing polarity. The eluate was separated into 71 fractions; these fractions were grouped in 20 fractions after TLC analysis (PB_1–PB_{20}). In MeOH, crystals precipitated in fraction PB_{14} (5.6896 g). These crystals were separated and then purified by recrystallization in hot methanol (MeOH) followed by $CHCl_3$ at room temperature, yielding poligalen (2.6913 g – 0.92% of chloroform portion).

2.4. Peritoneal macrophage culture

Peritoneal macrophages were harvested from euthanized C57BL/6 male mice by washing the peritoneal cavity with RPMI-1640 medium. The macrophage cell concentration was adjusted to 1×10^6/mL using medium RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (1 mg/mL) and glutamine (2 mM). Macrophages were seeded into a 96-well plate at 2×10^5 cells/well and incubated overnight at 37 °C in 5% CO_2. Before experiments, supernatants were replaced to remove nonadherent cells. Cells were the pre-treated with vehicle, poligalen, or dexamethasone 30 min prior to the addition of the lipopolysaccharide (LPS) stimulus (Sigma). After 4 h of incubation with LPS, supernatants were removed and stored at −70 °C until cytokine quantification via Enzyme-Linked Immunosorbent Assay (ELISA).

2.5. Quantification of TNF and IL-6

To measure the levels of TNF and IL-6 released into culture supernatants, we used the Duo Set Kit to perform ELISA according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). Concentrations are expressed in pg/mL.

2.6. MTT assay

Cell viability was assessed using the MTT assay adapted from the procedure described previously by Mosmann [19] and Silva et al. [20]. Peritoneal macrophages were treated as described above (3.5, Peritoneal macrophage culture). After incubation with treatments, supernatants were removed, and adherent cells were incubated with an MTT solution (1 mg/mL) (tetrazolium salt; Sigma) in RPMI-1640 medium for 2 h. Intracellularly accumulated MTT formazan was solubilized in dimethyl sulfoxide (DMSO) for 30 min with stirring. MTT reduction was quantified by measuring spectrophotometric absorbance at 590 nm. The results express the percentage of reduction compared to the vehicle-treated group.

2.7. Luciferase-nuclear factor kappa B (NF-κB) reporter assay

To analyze the effect of poligalen on NF-kB, RAW 264.7 macrophages that stably bear a luciferase reporter gene controlled by an NF-κB-sensitive promoter (pNF-κB-Luc) were used. The assay was performed as described by Silva et al. [20]. Briefly, cells were pre-treated with vehicle, poligalen or dexamethasone for 30 min and then incubated with LPS for 4 h. Cells were lysed with Tris-NaCl-Tween buffer (TNT-buffer), and sample luminescence was measured using Luciferase Assay Reagent (Promega, Madison, WI, USA) in a luminometer (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA). Data are expressed as luminescence units relative to the control group (Veh/LPS = 100).

3. Results and discussion

The poligalen (Fig. 1.) was obtained from chloroformic extract of the aerial parts and roots of *P. boliviensis* after chromatographic procedures.

Poligalen is a white crystalline solid with a melting point of 222–224 °C. The molecular formula was identified as $C_{14}H_{12}O_5$ by analyzing

Table 1
^{1}H and ^{13}C NMR shifts and two-dimensional correlations of poligalen[a].

Position	δ_H	HMBC	δ_C
2	–		159.9
3	6.45(*d*; 9.5 Hz)	159.9; 117.3	116.1
4	7.75 (*d*; 9.5 Hz)	159.9; 133.3	143.9
5	7.62 (*d*; 8.5 Hz)	117.3; 154.2; 156.4	133.3
6	6.96 (*d*; 8.5 Hz)	156.4	120.2
7	–	–	156.4
8	–	–	114.8
9	–	–	154.2
10	–	–	117.3
1′	–	–	164.7
3′	4.19 (*s*)	164.7	71.5
4′	–	–	84.7
6′	1.35 (*s*)	24.1; 84.7; 71.5	24.1
7′	1.35 (*s*)	24.1; 84.7; 71.5	24.1

[a] Spectra were run in pyridine-d5 at 500 MHz (^{1}H) and 125 MHz (^{13}C). *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm.