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Original article

Optimization of xanthatin extraction from *Xanthium spinosum* L. and its cytotoxic, anti-angiogenesis and antiviral properties

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Dedicated to the Professor Rosa Maria Claramunt on the occasion of this 65 birthday

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ABSTRACT

The aqueous extraction of the sesquiterpene lactone xanthatin from *Xanthium spinosum* L. favours the conversion of xanthinin (**1**) to xanthatin (**2**) via the loss of acetic acid. The cytotoxic (Hep-G2 and L1210 human cell lines) and antiviral activities of isolated xanthatin are established. This natural compound shows significant cytotoxicity against the Hep-G2 cell line and our experimental results reveal its strong anti-angiogenesis capacity *in vitro*. The structure of xanthatin is determined by spectroscopic methods and for the first time confirmed by X-ray diffraction.

1. Introduction

The plant *Xanthium spinosum* L. (Asteraceae, Compositae) has been the object of a large number of chemical and biological studies. It is used in traditional medicine in different countries [1,2]. Extracts from *X. spinosum* L. contain a mixture of compounds such as: β-sitosterol, quercetin and other flavonoids such as pendulin, iocein, centaurin and patuletin 3-*O*-glucoside [3]. Moreover, it contains xanthanolide sesquiterpene lactones such as: xanthinin (**1**), xanthatin (**2**), stizolicin (**3**) and solstitialin (**4**) [4] (Fig. 1).

The chloroform extracts of *X. spinosum* aerial parts are reported to be used to treat hydrophobia treatment, rabies and intermittent fever [5] as well as for the treatment of diarrhoea and cancer [3,6]. The isolated sesquiterpene lactones xanthinin (**1**), stizolicin (**3**) and solstitialin (**4**) were tested for antitumor action *in vitro* on two types of tumors: L-1210 leukemia and P-388 leukemia [7].

The major constituents of *X. spinosum* dichloromethane extraction showed antiviral properties [8,9] whereas the methanol extracts of *Xanthium strumarium* leaves have been used to treat inflammatory diseases such as rheumatoid arthritis [10] and have also showed anticancer activity with $IC_{50} = 3.0$, 2.2 and 1.5 μg/mL against MCF-7, A431 and HepG2 cell respectively [11]. The main responsible constituents of the inhibition of the proliferation of human tumor cells *in vitro* were the xanthanolides 8-epixanthatin and 8-*epi*-xanthatin epoxide [12].

Xanthanolides are a class of sesquiterpenoids isolated primarily from Xanthium (family Asteraceae) [13]. The chemical structures of numerous xanthanolides are well documented, though much more is known of the biological data of the extracts than of their synthetic preparation. In general, the xanthanolides present a γ-butirolactone fused to a seven-membered ring. Depending of this fusion, the xanthanolides can be divided into two structural types:

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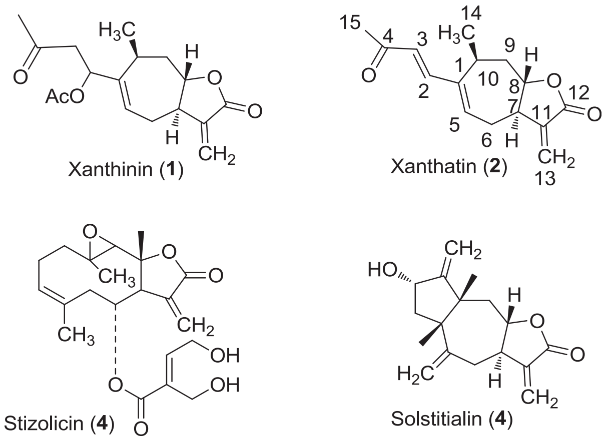

Fig. 1. Sesquiterpene lactones.

the *cis*- (8-*epi*-xanthatin, xanthanolide numbering) and the *trans*-fused lactones (Fig. 1). Xanthatin (**2**) was first isolated from *Xanthium pennsylvanicum* by J. E. Little and co-workers [14] and its structure was established by Geissman et al. [4,15]. The latter group suggested the possibility that xanthatin could be formed from xanthinin by dehydration during isolation, particularly during purification by column chromatography. The first total synthesis of a xanthanolide, specifically (−)-dihydroxanthatin, was reported by Morken with sequential ruthenium-catalysed metathesis reactions [16]. Recently, Shishido and co-workers reported the enantiocontrolled total synthesis of (−)-xanthatin from an optically pure bicyclic lactone [17].

Sesquiterpene lactones exhibit interesting biological activities not exploited by the pharmaceutical industry and have also attracted considerable attention from organic chemists interested in synthetic routes to produce these natural products and for their structural characterization.

We use an extraction process to obtain **2** from *X. spinosum* and evaluate its anticancer, antiviral and anti-angiogenic activities. Its structure was established by ^{1}H NMR, ^{13}C NMR, IR and MS, and confirmed by X-ray crystallography for the first time (The resulting crystal structure has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 755280) [18].

2. Material and methods

2.1. General methods

Melting points were obtained on an MFB-595010M Gallenkamp apparatus in open capillary tubes and are uncorrected. IR spectra were obtained using an FTIR Perkin–Elmer 1600 Infrared Spectrophotometer. Only noteworthy IR absorptions are listed (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 (200 and 50.3 MHz respectively) or Varian Gemini-300 (300 and 75.5 MHz) Instrument using $CDCl_3$ as solvent and tetramethylsilane as internal standard or $(CD_3)_2CO$. Other ^{1}H NMR spectra and heterocorrelation ^{1}H–^{13}C (HMQC and HMBC) experiments were recorded on a Varian VXR-500 (500 MHz). Assignments were established by DEPT, HMBC and HMQC. Mass spectra were recorded on a ThermoFinnigan Trace DSQ equipped with an APCI or ESI source. High-resolution ESI-MS were measured on an LC/MSD-TOF mass spectrometer. Internal reference masses $m/z = 121.050873$ (purine), 922.009798 (HP-0921), data acquisition and processing were performed with Xcalibur 1.3 software. Column chromatography was performed with silica gel (E. Merck, 70–230 mesh). Reactions were monitored by TLC using 0.25 mm silica gel 60 F_{254} plates (E. Merck), detection was achieved by the absorbance of UV light (254 nm and 366 nm) or spraying with different reagents (sulfuric acid-10% w/w ceric sulfate followed by heating 5 min at 80–110 °C depending of the used reagent) and the R_f values was obtained from the analytical TLC. Microanalysis was determined on a Carlo Erba–1106 analyser. All reagents were of commercially quality or were purified before use. Organic solvents were of analytical grade or were purified by standard procedures. Commercial solvents were purchased from Sigma–Aldrich.

2.1.1. Materials

DMEM (Dulbecco's modified Eagle's medium, Sigma, St. Louis MO, USA), PBS (phosphate saline buffer, Sigma, St. Louis MO, USA), DMSO (dimethylsulfoxide, Sigma, St. Louis MO, USA), FCS (fetal calf serum, Gibco-BRL, Eggenstein, Germany), penicillin/streptomycin (Gibco-BRL, Eggenstein, Germany), culture well-plates (Techno Plastic Products, Trasadingen, Switzerland), MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis MO, USA).

2.1.2. Drugs

The product extracted was previously dissolved in 10 mg/mL in DMSO. In the treatment, this solution was diluted in DMEM containing 10% FCS and 0.1% penicillin/streptomycin.

2.2. Plant material

Aerial fresh young plants of *X. spinosum* (Asteraceae family) were collected in June and July at Barcelona coastline (Spain) and were authenticated by comparison with a sample of this specie deposited at the Institut Botanic, CSIC-Ajuntament de Barcelona (Spain). The species were air-dried and ground.

2.3. Extraction and isolation

A dry residue (100 g) of *X. spinosum* L. was soaked in 300 mL of water. The mixture was heated at 100 °C in an ultrasound bath for 30 min. Then, the residue was filtered under suction and the filtrate was extracted with ethyl ether: ethyl acetate, 1:1, in 3 portions. The solid residue was discarded and the combined organic extracts were dried over Na_2SO_4, filtered and the solvents removed under vacuum.

The residue obtained from the evaporation of the solvents was satisfactorily purified by column chromatography using a column 50 cm high place with a diameter of 2.5 cm which was packed with slurry of silica gel and hexane. The residue was dissolved in the minimum amount necessary of the dichloromethane and applied with a pipette to the top of the silica bed. The column was eluted with a hexane-ethyl acetate mixture of increasing polarity, as the solvent system. The solvent gradient used ranged from 100% hexane to 100% ethyl acetate and fractions of 15 mL were collected. The fractions were collected manually and those with similar TLC profiles were pooled. Xanthatin was eluted with hexane-ethyl acetate, 1:1, and caffeic acid was obtained by elution with ethyl acetate 100%.

The xanthatin extracted from *X. spinosum* is a white solid that is soluble in chloroform, dichloromethane, methanol and acetone; and slightly soluble in cold water. The xanthatin obtained was purified by column chromatography using silica gel to 99.9% purity before the analytical and biological tests.