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

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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: b-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 IC50 ¼ 3.0, 2.2 and 1.5 mg/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 gbutirolactone fused to a seven-membered ring. Depending of this fusion, the xanthanolides can be divided into two structural types: the cis- (8-epi-xanthatin, xanthanolide numbering) and the transfused 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 1H NMR, 13C NMR, IR and MS, and confirmed by X-ray crystallography for the first time (The resulting crystal structure has been deposited

82 at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 755280)
83 [18].
84

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 PerkineElmer 1600 Infrared Spectrophotometer. Only noteworthy IR absorptions are listed (cm 1). 1H and 13C 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 CDCl3 as solvent and tetramethylsilane as internal standard or (CD3)2CO. Other 1H NMR spectra and heterocorrelation 1He13C (HMQC and HMBC) experiments were recorded on a Varian VXR-500 (500 MHz). Assignments were established by DEPT, HMBC and HMQC. Mass spectrawere 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 ¼ 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, 70e230 mesh). Reactions were monitored by TLC using 0.25 mm silica gel 60 F254 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 80e110 C depending of the used reagent) and the Rf values was obtained from the analytical TLC. Microanalysis was determined on a Carlo Erbae1106 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 SigmaeAldrich.

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.

122 2.2. Plant material
123 Aerial fresh young plants of X. spinosum (Asteraceae family) were collected in June and July at
124 Barcelona coastline (Spain) and were authenticated by comparison with a sample of this specie
125 deposited at the Institut Botanic, CSIC-Ajuntament de Barcelona (Spain). The species were air-dried and
126 ground.
127
128 2.3. Extraction and isolation
129 A dry residue (100 g) of X. spinosum L. was soaked in 300 mL of water. The mixture was heated at 100
130 C in an ultrasound bath for 30 min. Then, the residuewas filtered under suction and the filtrate was
131 extracted with ethyl ether: ethyl acetate, 1:1, in 3 portions. The solid residue was discarded and the
132 combined organic extracts were dried over Na2SO4, filtered and the solvents removed under vacuum.
133 The residue obtained from the evaporation of the solvents was satisfactorily purified by column
134 chromatography using a column 50 cm high place with a diameter of 2.5 cm which was packed with
135 slurry of silica gel and hexane. The residue was dissolved in the minimum amount necessary of the
136 dichloromethane and applied with a pipette to the top of the silica bed. The column was eluted with a
137 hexane-ethyl acetate mixture of increasing polarity, as the solvent system. The solvent gradient used
138 ranged from 100% hexane to 100% ethyl acetate and fractions of 15 mL were collected. The fractions
139 were collected manually and those with similar TLC profiles were pooled. Xanthatin was eluted with
140 hexane-ethyl acetate, 1:1, and caffeic acid was obtained by elution with ethyl acetate 100%.
141 The xanthatin extracted from X. spinosum is a white solid that is soluble in chloroform,
142 dichloromethane, methanol and acetone; and slightly soluble in cold water. The xanthatin obtained was
143 purified by column chromatography using silica gel to 99.9% purity before the analytical and biological
144 tests.
145 Xanthatin: CA: 26791-73-1. IUPAC name: 3,3a,4,7,8,8a-hexahydro-7-methyl-3-methylene-6-((E)-3-
146 oxo-1-buten-1-yl)-(3aR,7S,8aS,(E))-2H-cyclohepta[b]furan-2-one. The structure was established by
147 spectral methods and confirmed by X-ray diffraction. Analytical data was also compared with the
148 bibliographic reported data [4]. White needles (methanol); mp 111e113 C (111e112 C) [4],
149 114.5e115 C (Merck Index 2006, compound number 10058); Rf 0.39, silica gel 60 F254, hexane:ethyl
150 acetate (1:1);½a 20 D ¼ 20 (c ¼ 0.12 g in 5 mL of CH2Cl2) [4], IR (KBr) ymax 1763 (C]O), 1683
151 (C]O), 1586 (C]CH), 976 (CeO), 896 (C]CeC) cm-1; EM (IE) (m/z, %), 246 (Mþ, 15), 231 (M 15, 18),
152 204 (47), 175 (75), 123 (100), 91 (C7H7þ, 97), 77 (C6H5þ, 90), 53 (C4H5þ, 94); anal. C 73.44%, H
153 7.09%. calcd. for C15H18O3, C 73.15%, H 7.37%.
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159 2.5. In vitro cytotoxic activity assay

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161 2.5.1. Cell culture

162 This study was performed on human hepatic cellular carcinoma Hep-G2 cells (ECACC n. 85011430).
163 The cells were cultured in DMEM supplemented with 2 mM glutamine, 10% heat-inactivated FCS (fetal
164 calf serum), 10,000 units/mL of penicillin and 10 mg/mL of streptomycin. The cells were maintained at
165 37 ° C in vitro as monolayer cultures placed in 100 cm diameter culture well-plates in a 95% humidified
166 atmosphere with 5% CO2. After confluence, the cells were subcultured following trypsinization and
167 centrifugation every 3e4 days, depending on the number of cell. Cell viability for the different
168 experiments and cell counts were assessed by trypan blue (0.04%) exclusion dye using a
169 haemocytometer. The drugs to be tested were added to cultures one day after seeding the cells to ensure
170 uniform attachment of the cells at the beginning of the experiments [19].

171

172 2.5.2. MTT assay

173 To determine of antiproliferative activity on tell cell lines, the viability of the cultured cells was
174 determined by assaying the reduction of MTT: the cells with metabolic capacity reduce MTT to
175 formazan. The assay was performed by a variation of the method described by Mosmann [20], 5103
176 cells/well were seeded in 96 well plates with 200 mL medium. After 24 h incubation at 37 ° C, to ensure
177 cell adherence, the mediumwas removed and new medium was added containing different
178 concentrations of the compounds. The cells were incubated for 24 h at 37 ° C in a humidified incubator
179 with 5% CO2. After incubation, MTT was added to a final concentration of 0.5 mg/mL in the medium.
180 The cells were incubated for 1 h at 37 ° C. Then 100 mL of DMSO was added per well to dissolve the
181 formazan crystals. Relative cell viability was obtained by measuring the absorbance on an ELISA plate
182 reader (Tecan Sunrise MR20-301, TECAN Austria) at 550 nm. The absorbance is directly proportional
183 to the level of cell proliferation or viability [19]. The IC50 value was determined from a doseeresponse
184 curve using 4 different concentrations. Each concentration was analysed in triplicate.

185

186 2.6. In vitro antiviral activity assay

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188 The antiviral activity of xanthatin was evaluated against the following viruses: herpes simplex virus type
189 1 (HSV-1); herpes simplex virus type 2 (HSV-2); varicela-zoster virus (VZV); vesicular stomatitis virus;
190 vaccinia virus; feline corona virus (FIPV); feline herpes virus; coxsackie virus B4; respiratory syncytial
191 virus; influenza A H1N1; influenza A H3N2; influenza B; parainfluenza-3 virus; repvirus-1; sindbis
192 virus and punta toro virus
193 The anti-virus assay method has been described previously. The antiviral activity was evaluated based
194 on the inhibition of virus induced cytopathicity or plaque formation in human embryonic lung (HEL)

195 fibroblasts, African green monkey cells (VERO), human epithelial cells (HeLa) or in Crandell-Rees
196 feline kidney cells (CRFK).
197 Human embryonic lung (HEL) fibroblasts were grown in 96-well microtitre plates infected with the
198 corresponding virus and incubated. After a 2 h incubation period, the residual virus was removed and
199 the infected cells were further incubated with the medium containing different concentrations of
200 xanthatin (in duplicate). After 6 days, the cell cultures were examined microscopically. Antiviral activity
201 was expressed as the 50% effective concentration required to reduce viral plaque formation after 5 days
202 by 50% compared to untreated controls.
203
204 2.7. In vitro angiogenesis activity assay
205
206 Freshly cut aortic rings obtained from 5- to 10-week-old Fischer 344 male rats were embedded in
207 collagen gels and transferred to 16mmwells each containing 0.5 mL of MCDB 131 as described [21].
208 Controls were treated with vehicle alone (0.2% dimethylsulfoxide (DMSO)). The microvascular growth
209 curves are characteristic for each gel [22e25]. Experiments included minimum three to four observations
210 per data point and were repeated at least two times.
211
212 2.8. Statistical analysis
213
214 Statistical calculations were carried out with the Microsoft Office Excel 3.0. Results are expressed as the
215 mean ± S.E.M. of minimum 5 independents experiments. Student's t-test was used for statistical
216 analyses and P values <0.05 were considered to be significant.
217

3. RESULTS AND DISCUSSION

3.1. Chemistry

Xanthatin has previously been extracted from several genera of Xanthium and other genera of the Asteraceae family using organic solvents (acetone [15], dichloromethane, methanol [12] and others) but not by aqueous reflux. Therefore, Geissman and co-workers converted xanthinin to xanthatin by treatment with sodium acetate in ethanol [15]. In this work, to determine the principal constituents of infusions of X. spinosum, extraction was performed by boiling the dry extract in water for a short period of time, assisted by ultrasound. The crude residue represented 24% of the dry weight of fresh plants of X. spinosum used as the starting material. This simple process requiring little time (30 min), mild temperatures (<100 ° C) and soft frequencies (ranging from 20 to 50 kHz) is very efficient. These conditions favour the conversion of xanthinin (1) to xanthatin (2), and this would explain the formation of xanthatin (2) and no detection of xanthinin (1) or its derivatives.

Caffeic acid was obtained as 1.12e1.21% of the dry plant weight. From each sample of 100 g of plant powder 1.1e1.3 g of xanthatin (2) was obtained. This represents 1.1e1.3% of the dry plant weight or 0.9e1.04% of the fresh plant weight (quantities higher than those published in other works). An infusion with 1.5 g of dry plant contains approximately 16.5e19.5 mg of xanthatin (2) and 16.8e18.2 mg of caffeic acid. With this extraction procedure, no other sesquiterpenes were isolated.

For the structural study 1H NMR, 13C NMR and 2D 1He13CHeteronuclear shift correlation spectra were used. The relative stereochemistry of xanthatin (2) was determined for the first time by the X-ray diffraction (3aR*, 7S*, 8aS*). Thus, whereas H-3a is in the a position, H-8a is in the b-position. The configuration of double bonds C5eC6 and C10-C20 is (E). Five olefinic protons were observed in the 1H NMR: two trans-coupled protons at 6.20 and 7.08 ppm (in CDCl3) with a J ¼ 16 Hz, corresponding to H-10 and H-20, two coupled protons at 5.50 and 6.21 ppm with a J ¼ 3.4 Hz corresponding to the exocyclic methylene and a methyne group at 6.27 ppm corresponding to the H-5. The HMQC and HMBC analysis confirmed the position of methyl and methylene groups and also the presence of a,b-unsaturated ketone at C-6 position of the heterocyclic ring.

The presence of the g-butirolactone condensed with a cycloheptene ring, the position of the side chain and the a-exocyclic methylene group at C-3 position were confirmed from the singlecrystal X-ray crystallographic analysis (Fig. 2). All the analytical data were verified by the comparison with these reported in the literature [4,15]. From the structural evidence, the main compound isolated was identified as xanthatin (2).

255 3.2. Pharmacology
256
257 The cytotoxicity of xanthatin (2) in Hep-G2 (hepatocellular carcinoma, human) and L1210 (mouse
258 lymphocytic leukemia) cell cultures was evaluated. Their antiviral properties and capacity to inhibit
259 angiogenesis were also tested.
260
261 3.2.1. Cell growth inhibition
262 The cytotoxic properties of Xanthium (Asteraceae) are well known, but the in vitro cytotoxicity of
263 isolated xanthatin (2) has been less studied [26,27]. Inhibition of cell proliferation was observed
264 following treatment of Hep-G2 and L1210 cell lines with xanthatin. The concentration required for 50%
265 inhibition of growth, IC50 at 24 h on Hep-G2 cells was 49.0 ± 1.2 mM (Fig. 3) and 12.3 ± 0.9 mM on
266 L1210 cells. The relative percentage of cell proliferation was calculated by considering untreated control
267 cells after 24 h as 100% cell proliferation.
268
269 3.2.2. Antiviral activity
270 Xanthatin (2) showed inhibitory activity against a wide variety of virus including Herpes simplex,
271 vaccinia and vesicular stomatitis in HEL cell cultures, feline corona, feline herpes in CRFK cell cultures,
272 vesicular stomatitis virus, coxsackie virus B4 and respiratory syncytial virus in HeLa cell cultures. The
273 results were compared to these obtained for the reference compounds brivudin, ribavirin, ciclofovir and
274 ganciclovir [28].
275 Xanthatin lacked inhibitory activity against the three types of influenza tested (influenza A H1N1,
276 Influenza A H3N2 and Influenza B) (Table 1). Xanthatin showed pronounced cytotoxic activity against
277 MDCK cell cultures of influenza and also against Vero cell cultures with minimal cytotoxic
278 concentrations (MCCs) of 4 and >20 mM respectively (Table 2).
279 This natural compound possesses a relatively high toxicity. The therapeutic index (CC50/EC50) is <5
280 for several virus tested and MCCs values where in the same range as their EC50 values.
281
282 3.2.3. Anti-angiogenesis activity
283 The formation of neovessels from the endothelium of pre-existing vessels is an essential part of
284 embryonic development and, tissue repair and plays a critical role in the progression of several fatal
285 diseases including cancer, diabetes, and rheumatoid arthritis [29]. The vasculature density is correlated
286 with the malignancy and aggressiveness of tumor. Angiogenesis favours the growth of solid tumors as
287 well as their invasion and metastasis [30,31]. Clinical results for the first generation of anti-angiogenic
288 drugs demonstrated no additional clinical benefit compared with the classic antitumor treatments.
289 Consequently, more research will be needed to obtain more promising angiogenesis inhibitors. The rat
290 aortic ring explants model in three-dimensional collagen gel matrixes and the HUVEC capillary tube
291 formation assay on a Matrigel synthetic basement membrane matrix are commonly used in vitro

292 angiogenesis systems. The anti-angiogenic response to xanthatin was evaluated in vitro in rat aorta ring
293 explant cultures over 14- days in the presence of exogenous VEGF stimulation. Doserelated inhibitory
294 effects on microvessel growth were observed from 0.03 mMto 10 mM(Fig. 4) and 0.3 nM to 3
295 mMxanthatin (Fig. 5).
296 Xanthatin displayed significant inhibition (IC50 ¼ 0.028 ± 0.001 mM) of capillary tube formation (by
297 measuring the area in mm2 with regard to the concentration of xanthatin tested) relative to untreated
298 control. This anti-angiogenic response was comparable to the effect of paclitaxel (IC50 ¼ 0.002 mM)
299 which is considered to be one of the best antiangiogenic drugs. Xanthatin and paclitaxel showed similar
300 behaviour. Thus, the anti-angiogenic effects of xanthatin and paclitaxel prevailed at low doses, while at
301 high doses both showed cytotoxic activity. Moreover, the inhibition of angiogenesis is not
302 dosedependent for either compound. The inhibition of angiogenesis has not previously been studied
303 either for xanthatin or for xanthium extract, and these properties could be used in the treatment of solid
304 tumor.
305

4. CONCLUSIONS

In conclusion, although xanthatin has been isolated from several species in the Asteraceae family, this is the first report of its fast aqueous extraction from X. spinosum and also the first X-ray confirmation of the structure proposed by Geissman and coworkers. The procedure allows xanthinin to be transformed into xanthatin and both the performance and the purity of this compound to be increased. Xanthatin shows cytotoxicity against L1210 and Hep-G2 cell lines. Excellent inhibition of angiogenesis was observed; comparable to that of paclitaxel. Also, xanthatin shows inhibitory activity against several viruses, but with a therapeutic index <5.

Xanthatin can be isolated from X. spinosum in small quantities and total synthesis is often difficult; but the isolated compound could be used as a lead compound for the development of xanthatin derivatives with therapeutic effects. The present study suggests that xanthatin plays an important role in angiogenesis inhibition, which is promising for tumor treatment. Further research is required to elucidate the mechanism of action of this class of compounds.

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323

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REFERENCES

[1] Ridvan Polat, Ugur Cakilcioglu, Fatih Satil, Traditional uses of medicinal plants in Solhan (Bing€oldTurkey), J. Ethnopharmacol. 148 (2013) 951e963 and references cited therein.

[2] D.M. Gonz alez Torres, Catalogo de Plantas Medicinales (y Alimenticias útiles) usadas en Paraguay, vol. 12, Editora Litocolor S.R.L., Asunci on, Paraguay, 2009, pp. 456e457.

[3] A.H. Ansari, K.S. Dubey, 2-Desacetyl-8-epi-xanthumanol-4-O-b-D-galactopyranoside: the potential antitumor sesquiterpenoidal lactone from Xanthium spinosum bark, Asian J. Chem. 12 (2000) 521e526.

[4] T.A. Geissman, P. Deuel, E.K. Bonde, F.A. Addicott, Xanthinin: a plant-growthregulating compound from Xanthium pennsylvanicum. I, J. Am. Chem. Soc. 76 (1954) 685e687.

[5] I. Ciulei, E. Grigorescu, U. Stanescu, in: Medicala (Ed.), Planta Medicinale, Fitochimie, Fitoterapie, vol. 2, 1993, pp. 215e228. Bucuresti.

[6] A. Salinas, R.E.L. De Ruiz, S.O. Ruiz, Sterols, flavonoids, and sesquiterpenic lactones from Xanthium spinosum (Asteraceae), Acta Farm. Bonaer. 17 (1998) 297e300.

[7] E. Naidenova, I. Kolarova-Pallova, D. Popov, S. Dimitrova-Konaklieva, L. Dryanovska-Noninska, Isolation and obtaining of sesquiterpene lactones with antitumor properties e xanthinin, stizolicin, and solstitialin, Natl. Oncol. Cent. Med. Acad. 41 (1988) 105e106.

[8] a) R.H. Willians, F.B. Martin, E.D. Henley, H.E. Swanson, Inhibitors of insulin degradation, metabolism, Clin. Exp. 8 (1959) 99e113; b) E. Ginesta-Peris, F.J. García-Breijo, E. Primo-Yúfera, Antimicrobial activity of xanthatin from Xanthium spinosum L, Lett. Appl. Microbiol. 18 (1994) 206e208; c) P. Cu~nat, E. Primo, L. Sanz, R. Martínez-Pardo, Biocidal activity of some Spanish Mediterranean plants, J. Agric. Food Chem. 38 (1990) 497e500.

[9] W.C. Evans, Trease and Evans Pharmacognosy, sixteenth ed., W B Saunders, London, 2002, pp. 333e393.

[10] J.H. Yoon, H.J. Lim, H.J. Lee, H.D. Kim, R. Jeon, J.H. Ryu, Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 expression by xanthanolides isolated from Xanthium strumarium, Bioorg. Med. Chem. Lett. 18 (2008) 2179e2182.

[11] Y. Sato, H. Oketani, T. Yamada, K.I. Singyouchi, T. Ohtsubo, M. Kihara, M. Shibata, T.J. Higuti, A xanthalonide with potent antibacterial activity against methicillin-resistant Staphylococcus aureus, J. Pharm. Pharmacol. 49 (1997) 1042e1044.

[12] a) Y.S. Kim, J.S. Kim, S.H. Park, S.U. Choi, C.O. Lee, S.K. Kim, Y.K. Kim, S.H. Kim, S.Y. Ryu, Two cytotoxic sesquiterpene lactones from the leaves of Xanthium strumarium and their in vitro inhibitory activity on farnesyltransferase, Planta Med. 63 (2003) 375e377; b) L. Wang, J. Wang, F. Li, X. Liu, B. Chen, Y.X. Tang, M.K. Wang, Cytotoxic sesquiterpene lactones from aerial parts of Xanthium sibiricum, Planta Med. 79 (2013) 661e665.

[13] E. Naidenova, L. Dryanovska-Noninska, I. Kolarova-Pavlova, S. Dimitrova-Konaklieva, Sesquiterpene lactones in Xanthium strumarium Asteraceae(Compositae), Probl. Farmatsiyata 10 (1982) 39e45. CAN 98:68819.

367 [14] J.E. Little, M.W. Foote, D.B. Johnstone, Xanthatin: an antimicrobial agent from Xanthium
368 pennsylvanicum, Arch. Biochem. 27 (1950) 247e257.

369 [15] P. Deuel, T.A. Geissman, Xanthinin. II. The structures of xanthinin and xanthatin, J. Am. Chem.
370 Soc. 79 (1957) 3778e3781.

371 [16] M.A. Evans, J.P. Morken, Asymmetric synthesis of (e)-dihydroxanthatin by the stereoselective
372 Oshima-Utimoto reaction, Org. Lett. 7 (2005) 3371e3373.

373 [17] H. Yokoe, M. Yoshida, K. Shishido, Total synthesis of (e)-xanthatin, Tetrahedron Lett. 49
374 (2008) 3504e3506.

375 [18] M. Romero, M. Zanuy, M. Font-Bardia, M. D. Pujol, Cambridge Crystallographic Data. CCDC
376 755280.

377 [19] A. Sieuwerts, J.G.M. Klijn, H.A. Peters, J.A. Foekens, The MTT tetrazolium salt assay
378 scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures in vitro
379 for the assessment of growth characteristics, IC50-values and cell survival, Eur. J. Clin. Chem.
380 Clin. Biochem. 33 (1995) 813e823.

381 [20] T. Mosmann, Effect of 2- mercaptoethanol and mycostatin on guinea pig lymphocyte
382 transformation: interpretation of results depends on the method of calculation, J. Immunol.
383 Methods 65 (1983) 55e63.

384 [21] R.F. Nicosia, A. Ottinetti, Growth of microvessels in serum-free matrix culture of rat aorta. A
385 quantitative assay of angiogenesis in vitro, Lab. Invest. J. Tech. Meth. Pathol. 63 (1990)
386 115e122.

387 [22] R.F. Nicosia, J.A. Ottinetti, Modulation of microvascular growth and morphogenesis by
388 reconstituted basement membrane gel in three- dimensional cultures of rat aorta: a comparative
389 study of angiogenesis in matrigel, collagen, fibrin, and plasma clot, In Vitro Cell Dev. Biol. 26
390 (1990) 119e128.

391 [23] R.F. Nicosia, P. Belser, E. Bonanno, J. Diven, Regulation of angiogenesis in vitro by collagen
392 metabolism, In Vitro Cell Dev Biol 27 (1991) 961e966.

393 [24] R.F. Nicosia, S.V. Nicosia, M. Smith, Vascular endothelial growth factor, platelet-derived
394 growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro, Am. J.
395 Pathol. 145 (1994) 1023e1029.

396 [25] W.H. Zhu, A. MacIntyre, R.F. Nicosia, Regulation of angiogenesis by vascular endothelial
397 growth factor and angiopoietin-1 in the rat aorta model: distinct temporal patterns of
398 intracellular signaling correlate with induction of angiogenic sprouting, Am. J. Pathol. 161
399 (2002) 823e830.

400 [26] a) Y.S. Kim, J.S. Kim, S.E. Park, S. Choi, C. Lee, S.K. Kim, Y.K. Kim, S.H. Kim, S.Y. Ryu,
401 New alkaloids from Forsythia suspensa and their anti-inflammatory activities, Planta Med. 69
402 (2003) 375e377; b) M. Lavault, A. Landreu, G. Larcher, J.P. Bouchara, F. Pagniez, P. Le Pape,
403 P. Richomme, Antileishmanial and antifungal activities of xanthanolides isolated from
404 Xanthium macrocarpum, Fitoterapia 76 (2005) 363e366.

405 [27] C. Roussakis, I. Chinou, C. Vayas, C. Harvala, J.F. Verbist, Cytotoxic activity of xanthatin and
406 the crude extracts of Xanthium strumarium, Planta Med. 60 (1994) 473e474.

407 [28] S. De Castro, C. García-Aparicio, G. Andrei, R. Snoeck, J. Balzarini, M.J. Camarasa, S.
408 Vel azquez, 4-Benzyloxy-g-Sultone derivatives: discovery of a novel family of non-nucleoside
409 inhibitors of human cytomegalovirus and varicella zoster virus, J. Med. Chem. 52 (2009)
410 1582e1591.

411 [29] M. Milkiewicz, E. Ispanovic, J.L. Doyle, T.L. Hass, Regulators of angiogenesis and strategies
412 for their therapeutic manipulation, Int. J. Biochem.. Cell. Biol 38 (2006) 333e357.

413 [30] D. Bouïs, Y. Kusumanto, C. Meijer, N.H. Mulder, G.A.P. Hospers, A review on pro- and anti-
414 angiogenic factors as targets of clinical intervention, Pharm. Res. 53 (2006) 89e103.

415 [31] G. Bergers, K. Javaherian, K.M. Lo, J. Folkman, D. Hannahan, Effects of angiogenesis
416 inhibitors on multistage carcinogenesis in mice, Science 284 (1999) 808e812.

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418 **Legends to figures**

419

420 **Figure 1.** Sesquiterpene lactones.

421

422 **Figure 2.**. Chemical structure and ORTEP of xanthatin.

423

424 **Figure 3**. Cytotoxic activity of xanthatin (IC50) on Hep-G2 (left) and L1210 cells (right).

425

426 **Figure 4.** Inhibition of angiogenesis in rat aortic ring in response to anti-angiogenic treatment
427 (xanthatin) at concentrations of 0.03e10 mM after 14 days. Paclitaxel was tested in the same experiment
428 to provide comparative data.

429

430 **Figure 5.** Anti-angiogenesis activity of xanthatin at 0.3 nMe3 mM is not dose-dependent

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434 **FIGURE 1.**

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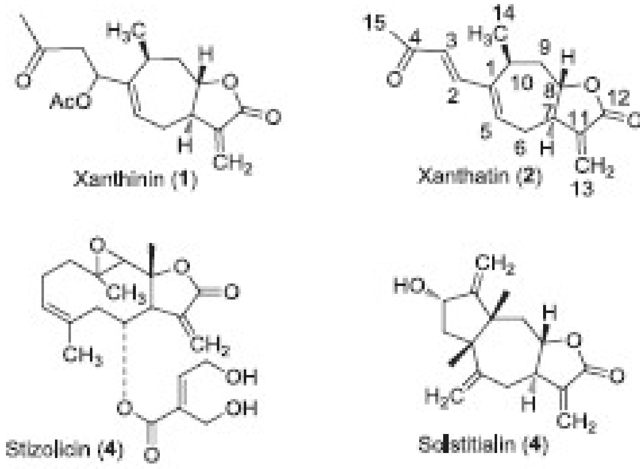

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439 **FIGURE 2.**

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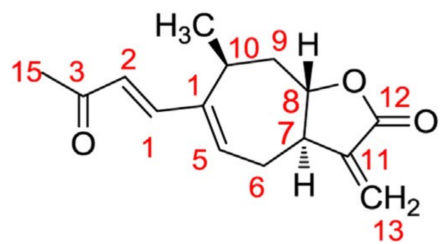

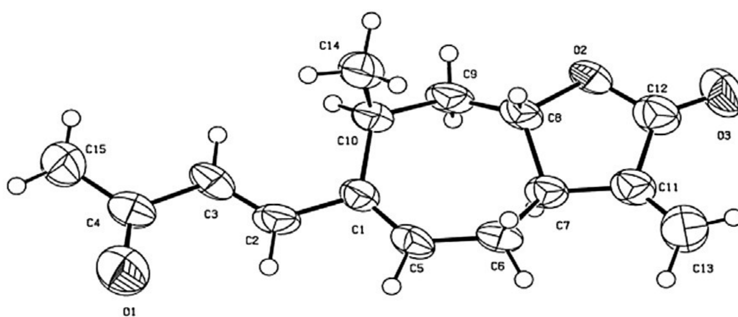

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445 **FIGURE 3.**

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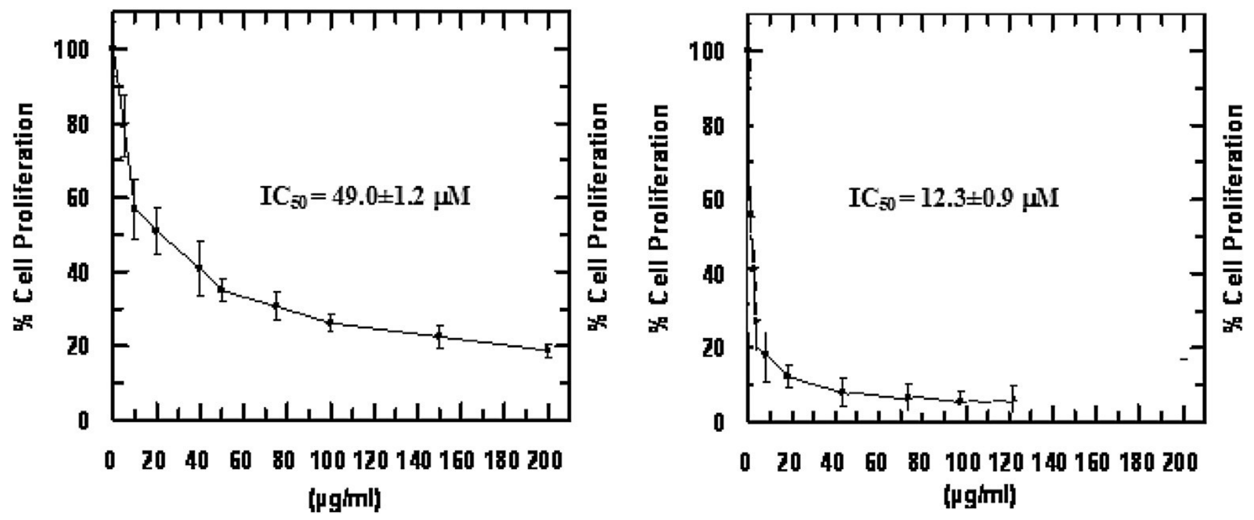

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450 **FIGURE 4.**

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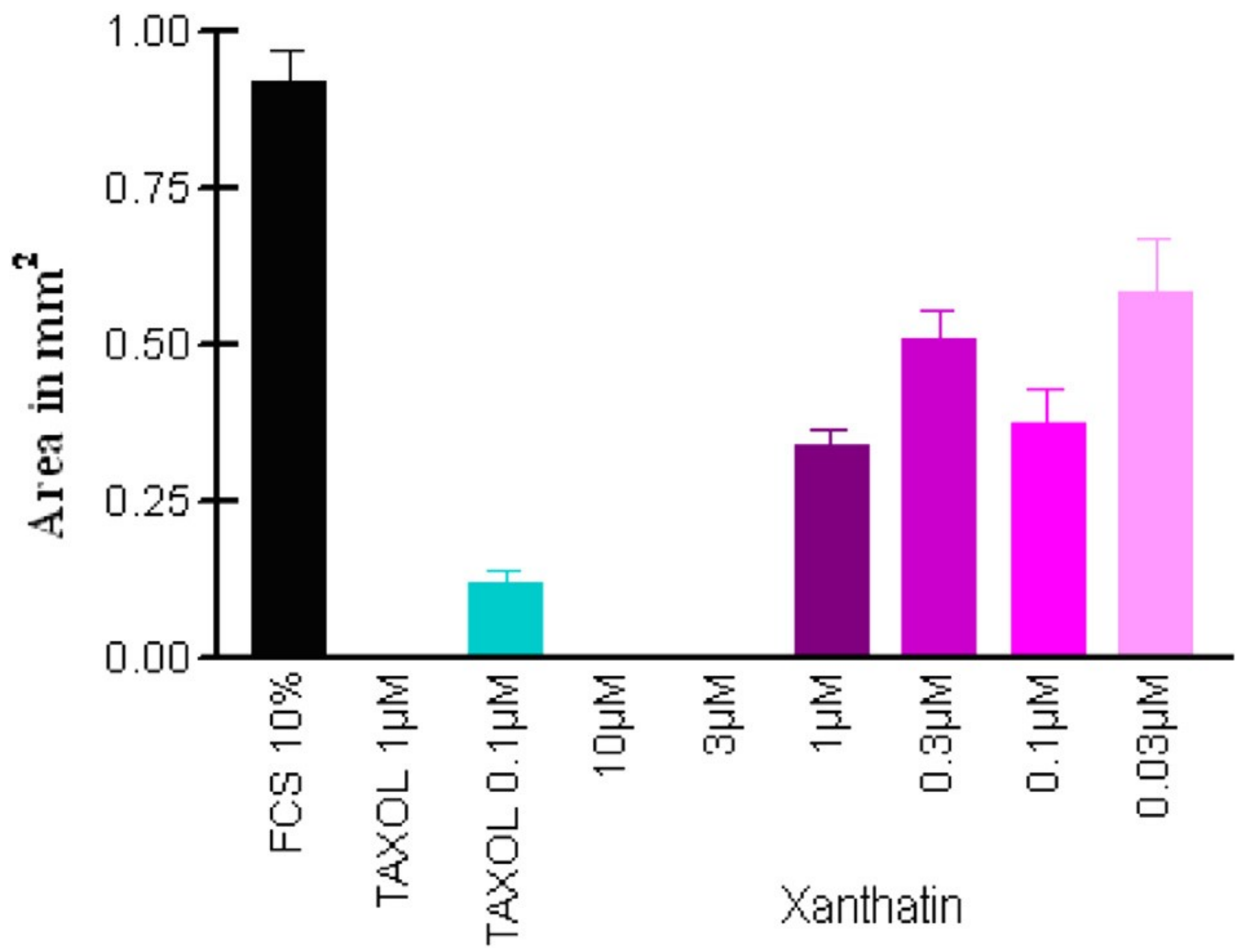

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455 **FIGURE 5.**

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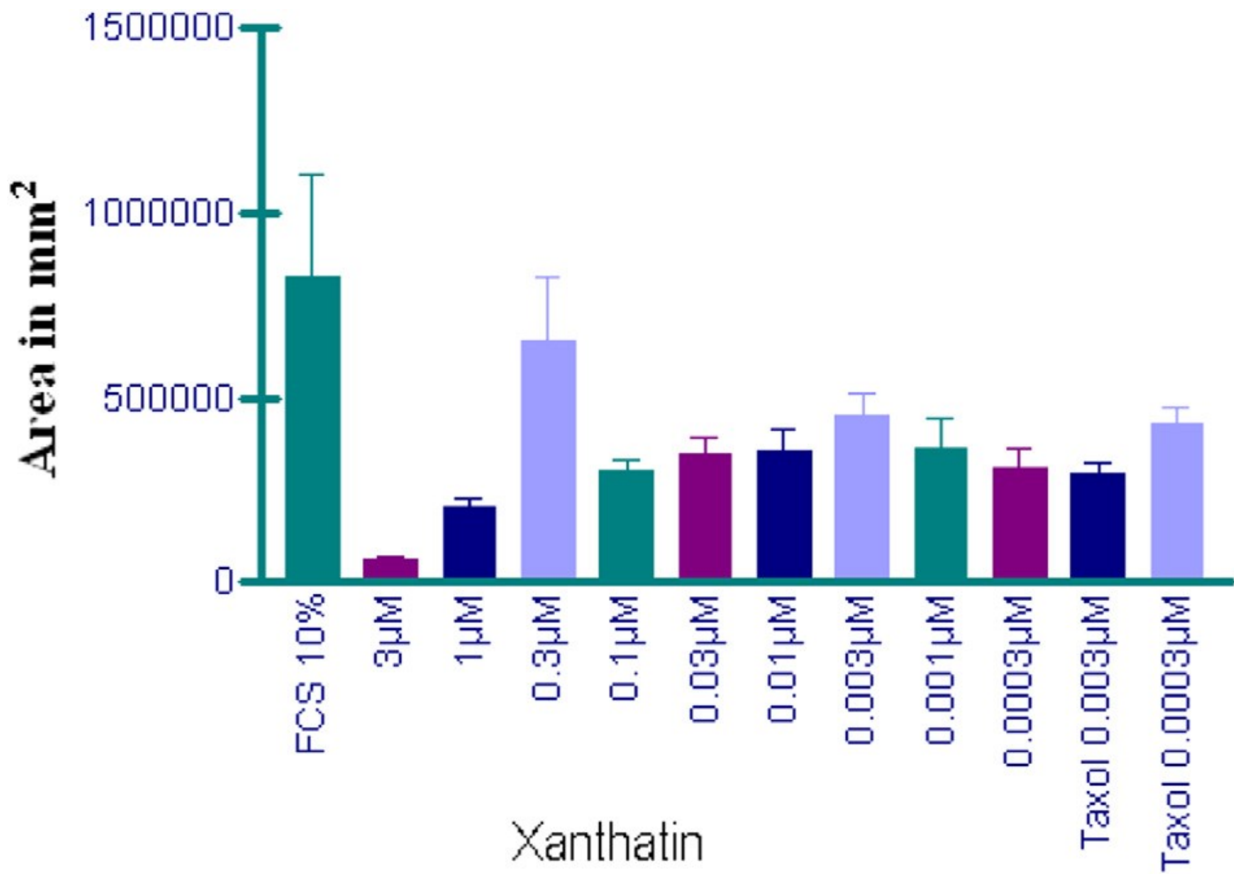

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460 **Table 1.** Anti-influenza virus activity and cytotoxicity in MDCK cell cultures.

461

Compound	Cytotoxicity		Antiviral EC_{50}[c]					
	CC_{50}[a] (μM)	Minimum cytotoxic concentration[b]	Influenza A H1N1 subtype		Influenza A H3N2 subtype		Influenza B	
			Visual CPE score	MTS	Visual CPE score	MTS	Visual CPE score	MTS
Xanthatin	1.6	4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Oseltamivir carboxylate	>100	>100	9 ± 0.22	13.6 ± 0.10	20 ± 0.61	18.2 ± 1.43	4 ± 0.11	6.2 ± 0.33
Ribavirin	>100	>100	9 ± 0.54	10.5 ± 0.65	8,9 ± 0.43	11.3 ± 0.78	9 ± 0.56	9.6 ± 0.98
Amantadine	>500	>500	34 ± 0.10	25.4 ± 0.54	20 ± 1.32	14.4 ± 0.55	N.A.	N.A.
Rimantadine	>500	>500	10 ± 0.27	9.2 ± 0.32	N.A.	N.A.	N.A.	N.A.

MDCK cells: Madin Darby canine kidney cells.
N.A.: not active at the highest concentration tested, or at subtoxic concentration.
[a] 50% cytotoxic concentration, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.
[b] Minimum compound concentration that causes a microscopically detectable alteration of normal cell morphology.
[c] 50% Effective concentration or concentration producing 50% inhibition of virus-induced cytopathic effect, as determined by visual scoring of the CPE, or by measuring the cell viability with the colorimetric formazan-based MTS assay.

462

463

464 **Table 2.** Cytotoxicity and antiviral activity of Xanthatin in Vero cell cultures.

465

Compound	Minimum cytotoxic concentration[a] (μM)	EC_{50}[b] (μM)				
		Para- influenza-3 virus	Reovirus-1	Sindbis virus	Coxsackie virus B4	Punta toro virus
Xanthatin	≥20	>20	>20	>20	>20	>20
DS-5000 (μg/mL)	>100	>100	>100	100	20 ± 2.3	73 ± 3.3
(S)-DHPA	>250	>250	>250	>250	>250	>250
Ribavirin	>250	112	>250	>250	>250	146 ± 7.8

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
466 [b] Required to reduce virus-induced cytopathogenicity by 50%.

467

468 **Table 3.** Crystallographic data for compounds 1 and 2

469

	1	2
Formula	$C_{10}H_{17}Cl_2N_3Zn$	$C_{22}H_{40}Cl_4N_6OZn_2$
Formula weight	315.53	677.14
Temperature [K]	293(2)	293(2)
Wavelength [Å]	0.71073	0.71073
System, space group	Monoclinic, $P2_1/c$	Orthorhombic, $Pna2_1$
Unit cell dimensions		
a [Å]	8.596(6)	23.177(10)
b [Å]	14.933(7)	8.481(5)
c [Å]	12.869(7)	15.229(5)
β [°]	121.95(4)	90
V [Å^3]	1495.7(15)	2993(2)
Z	4	4
D_c [g cm^{-3}]	1.500	1.502
μ [mm^{-1}]	2.112	1.986
$F(000)$	652	1400
Crystal size [mm^3]	0.2 × 0.09 × 0.08	0.2 × 0.1 × 0.1
h, k, l ranges	$-12 \le h \le 12, -22 \le k \le 20, -17 \le l \le 17$	$-34 \le h \le 34, -12 \le k \le 12, -22 \le l \le 20$
2θ range [°]	2.311 to 32.351	1.757 to 32.401
Reflections collected/unique/ (R_{int})	12674/4152 (R_{int} = 0.0827)	26213/9183 (R_{int} = 0.0646)
Completeness to θ [%]	94.6	99.3
Absorption correction	Empirical	Empirical
Max. and Min. transmission	0.5 and 0.5	0.82 and 0.79
Data/restrains/parameters	4152/2/145	9183/28/334
Goodness-of-fit on F^2	1.158	0.835
Final R indices ($I > 2\sigma(I)$)	$R_1 = 0.0555$, $wR_2 = 0.1167$	$R_1 = 0.0477$, $wR_2 = 0.0862$
R indices (all data)	$R_1 = 0.1014$, $wR_2 = 0.1307$	$R_1 = 0.1244$, $wR_2 = 0.1022$
Largest difference peak and hole [e Å^{-3}]	+0.459, −0.362	+0.975, −0.597

470

471

472 **Table 4.** Supramolecular interactions C–H...X (X5Cl or C) parameters for complexes 1 and 2

473

Complex	D–H···A	H···A [Å]	D···A [Å]	D–H···A [°]
1 (**L1**)	C4–H4B···Cl2 C4–H4C···Cl1	2.819, 2.913	3.688, 3.755	150.78, 166.96
2 (**L2**)	C9–H9B···pz ring	2.835	3.800	133.33
	Cl2···H–O1–H···Cl22 Cl11···H–O2–H···Cl12	2.633, 2.323	3.071, 3.372	122.11, 121.64

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