Planta Medica Thieme

Isolation, characterization and in vitro cytotoxicity of new sesquiterpenoids from Achillea clavennae

Journal:	*Planta Medica*
Manuscript ID:	PLAMED-2013-05-0502-OP.R2
Manuscript Type:	Original Papers
Date Submitted by the Author:	n/a
Complete List of Authors:	Trifunović, Snežana; Faculty of Chemistry, University of Belgrade, Isakovic, Andjelka; School of Medicine, University of Belgrade, Institute of Biochemistry Isakovic, Aleksandra; School of Medicine, University of Belgrade, Institute of medical and clinical biochemistry Vučković, Ivan; Faculty of Chemistry, University of Belgrade, Mandić, Boris; Faculty of Chemistry, University of Belgrade, Novakovic, Miroslav; Institute of Chemistry, Technology and Metallurgy, University of Belgrade , Vajs, Vlatka; Institute of Chemistry, Technology and Metallurgy, University of Belgrade , Milosavljević, Slobodan; Faculty of Chemistry, University of Belgrade, Trajkovic, Vladimir; School of Medicine, University of Belgrade, Institute of microbiology and immunology
Keywords:	Chemosystematics < Botany, Preparative Chromatography < Extraction, Isolation, Apoptosis < Molecular Targets and Activities, Cytotoxic, Cancer-related < Molecular Targets and Activities, GC-MS < Natural Products Analysis, MS < Natural Products Analysis, NMR < Natural Products Analysis, Sequiterpenes < Isoprenoids < Natural Products Classes

SCHOLARONE™
Manuscripts

Isolation, characterization and *in vitro* cytotoxicity of new sesquiterpenoids from *Achillea clavennae*

Snežana Trifunović[1], Anđelka M. Isaković[2], Aleksandra Isaković[2], Ivan Vučković[1], Boris Mandić[1], Miroslav Novaković[3], Vlatka Vajs[3], Slobodan Milosavljević[1] and Vladimir Trajković[4]

[1]Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia

[2]Institute of Medical and Clinical Biochemistry, School of Medicine, University of Belgrade, Pasterova 2, 11000 Belgrade, Serbia

[3]Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia

[4]Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia

Correspondence:

Dr. Snežana Trifunović, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia; E-mail: snezanat@chem.bg.ac.rs; Phone: +381 11 2630474; Fax: +381 11 2636061

Prof. Vladimir Trajković, Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr Subotića 1, 11000 Belgrade, Serbia; E-mail: vtrajkovic@med.bg.ac.rs; Phone: +381 11 3643233; Fax: +381 11 3643 235

For Peer Review

Abstract

Further phytochemical investigation into the aerial parts of *Achillea clavennae* has resulted in the isolation of three new sesquiterpene lactones: two highly oxygenated germacranolides (**1, 2**) and the *iso-seco*-guaianolide, 9(*R*)-acetoxy-3-*O*-methyl-*iso-seco*-tanapartholide (**3**). Eight known compounds were also found in this plant species, of which 9α-acetoxycanin (**5**), sintenin (**6**) and oleanolic acid (**7**) were detected for the first time. The structures of the isolated compounds were elucidated by combined spectroscopic methods (1D and 2D NMR, HRESIMS, CIMS, FTIR). While the predominant metabolite germacranolide sintenin (**6**) was not cytotoxic, the new *iso-seco*-guaianolide (**3**) displayed cytotoxicity comparable to that of cisplatin and the lactone apressin (**4**), inducing partly apoptotic death in human U251 and rat C6 glioma cell lines.

Key words: *Achillea clavennae* L. (Asteraceae), sesquiterpene lactones, *iso-seco*-guaianolide, NMR, cytotoxicity, apoptosis

For Peer Review

Introduction

The genus *Achillea* L. contains over 100 species, mainly distributed in Europe and the temperate areas of Asia [1]. Hitherto, about sixty *Achillea* species have been phytochemically investigated, but far fewer have been biologically tested [2-3]. The composition and antibacterial activities of the essential oils have been examined the most, even many times for same species, as in the case of *A. millefolium*, the most popular and wide-spread species of the genus. Alkamides from the roots were studied in detail, as compounds initially considered relevant for chemotaxonomy. Nowadays, the leaf exudates (mainly containing flavonoid aglycons) are most frequently analyzed for this purpose. The main constituents of the investigated plant extracts are terpenoids (largely sesquiterpene lactones) and flavonoids, classes of compounds with documented biological activity [2-3]. This fact, as well as the use of many *Achillea* species in traditional medicine worldwide (for their anti-inflammatory, antispasmodic, stomachic, digestive, cholagogue, antipyretic, emmenagogue, hemostatic, hypotensive, diuretic and urinary antiseptic activities), makes this genus interesting for extensive examination.

Achillea clavennae, which is the subject of the present study, grows on the elevated carbonate rocks of the eastern and southern Alps, as well as on mountains in the western part of the Balkan Peninsula (at altitudes between 1500 and 2500 m). According to the morphological characters and flavonoid profiles, *A. clavennae* belongs to the group of alpine species (the subgroup *A. moschata*) of the section *Ptarmica* (Mill.) W.D.J. [4]. This species was discovered and described as a healing plant (cholagogue, stomachic and anthelmintic) at the beginning of the 17th century [5]. At present, it is used in Croatian folk medicine to treat abdominal pain, the common cold, influenza and respiratory disorders [6]. Examination of the essential oil of *A. clavennae* from this locality verified its high antibacterial activity against some respiratory tract pathogens [6-7]. The

For Peer Review

extract of a plant collection from Macedonia (containing alkanes, fatty acids, monoterpenes, guaiane sesquiterpenes and flavonoids) also exhibited a wide range of antimicrobial activities [8]. Our previous study of the aerial parts of *A. clavennae* from Montenegro (the Komovi Mountain) revealed that guaianolides, bisabolenes and flavonoids were its main constituents. Subsequent work, which was the first assessment of the biological activity of *A. clavennae* constituents, revealed a remarkably high cytotoxic activity of the lactones 9α-acetoxyartecanin and apressin [9].

In the present study, the aerial parts of this alpine species from the Prokletije Mountains (Montenegro) were analyzed. Three new sesquiterpene lactones (**1-3**) and eight known compounds (apressin (**4**), 9α-acetoxycanin (**5**), sintenin (**6**), oleanolic acid (**7**), chrysetunone, 11-dehydroxy,11-hydroperoxyindicumenone, sesamin, centaureidin) were isolated and structurally determined. In addition, the *in vitro* cytotoxic activity of the predominant lactones **3, 4** and **6** was examined.

Results and Discussion

Fractionation of the crude extract (petrol-Et_2O-MeOH, 1:1:1) of the air-dried aerial parts of the title plant, by repeated silica gel column chromatography and preparative TLC, gave six sesquiterpene lactones (new lactones **1-3**, apressin (**4**) [10], 9α-acetoxycanin (**5**) [11] and sintenin (**6**) [12]), two bisabolenes (chrysetunone [13] and 11-dehydroxy,11-hydroperoxyindicumenone [9]), lignan sesamin [14], flavonol centaureidin [15], and oleanolic acid (**7**) [16] (**Fig. 1**). Apressin (**4**), chrysetunone**,** 11-dehydroxy,11-hydroperoxyindicumenone, sesamin and centaureidin had already been identified in the aerial parts of *A. clavennae* [9], but compounds **5,**

6, and **7** were found for the first time in this plant species. Oleanolic acid, atypical for the genus *Achillea*, had previously been reported only in *A. teretifolia* [17]. Bisabolene derivatives also rarely occur in this genus (*A. odorata* [18], *A. cretica* [19] and *A. clavennae* [9]), especially hydroperoxy-bisabolenes, previously found only in *A. clavennae*.

Fig. 1

The molecular formula $C_{19}H_{24}O_8$, for the lactone **1**, was deduced from the positive-ion mode HRESIMS (*m/z* 381.15398 $[M+H]^+$; calc. 381.15439). The CI mass spectrum showed the pseudomolecular ion, $[MH\text{-}O_2]^+$ (*m/z* 349), $[MH\text{-}O_2\text{-}HOAc]^+$ (*m/z* 289) and $[MH\text{-}2HOAc]^+$ (*m/z* 261) ions, indicating the presence of the hydroperoxy and two acetoxy groups. The germacranolide core of **1**, with C-4 and C-10(14) double bonds, α-methylene-γ-lactone group and the substitution at C-1, C-3 and C-9, was recognized in ^{1}H NMR spectrum (**Table 1**). Singlets at δ 2.03 and δ 2.08, and broad singlet at δ 8.75 originated from two OAc and HOO groups, respectively. Low-field signals at δ 5.33 *dd* (J_1 = 5.0 Hz, J_2 = 11.5 Hz), δ 5.14 *brd* (J = 10.5 Hz) and δ 4.24 *brd* (J = 10.0 Hz) belonged to their geminal protons: H-3, H-9 and H-1. H-3 was distinguished on the basis of HMBC correlations H-3(δ 5.33)/C-5 and H_3-15/C-3(δ 75.6). Carbons at δ 76.8 and δ 86.1, connected in the HSQC spectrum with protons at δ 5.14 and δ 4.24, respectively, showed HMBC correlations with H_2-14, which only proves C-9 and C-1 positions of remaining substituents. Other significant HMBC correlations regarding the arrangement of this part of molecule were missing. Taking into account the presence of the lactone sintenin in this plant sample (biogenetically related to **1**), and COSY and NOESY correlations (**Fig. 2**) of the proton geminal to OAc (δ 5.14), as well as of H geminal to OOH (δ 4.24), it was deduced that the

For Peer Review

former proton is H-9 and the latter is H-1. Moreover, NOEs H-9/ H-7, H-8α, H-1 and H-1/H-2α, H-3, H-5 revealed α-position of these protons, hence β-position of all substituents. One more high-frequency shifted *brd* (J = 10.5 Hz) at δ 5.45 was assigned to H-5 on the basis of HMBC correlations H_3-15,H-3/C-5(122.2). Due to the lack of the important HMBC correlations, coupling patterns in COSY spectrum and NOESY cross-peaks (**Fig. 2**) were crucial for distinguishing CH_2-2 and CH_2-8 groups, as well as identifying α and β protons. NOEs H-6/H_3-15 and H_3-15/H-14' disclosed *syn*-orientation of Me-15 and CH_2-14 on the β-face of the trans-4-cyclodecene. Such geometry affects spatial closeness of α-pseudo-axial protons H-1, H-3, H-5 and H-9, as well as of β-pseudo-axial H-2 with H-14' and Me-15, and also H-8β with H-14 and H-6, which was evidenced by corresponding NOE correlations (**Fig. 2**). Finally, **1** was identified as 3β,9β-diacetoxy-1β-hydroperoxy-6β,7αH-germacra-4,10(14),11(13)-trien-12,6α-olide. Three substituted $\Delta^{4,10(14)}$-germacranolides have not previously been found in the genus *Achillea*.

Fig. 2

For lactone **2**, closely related to **1**, the structure was determined from the positive-ion mode HRESIMS, CIMS, and ^{1}H NMR spectra. Prior to recording 2D NMR spectra, this compound was decomposed in the NMR tube, therefore the stereochemical data were obscured. According to $[M+H]^+$ ion at m/z 367.17513 (calc. 367.17485) in HRESIMS the molecular formula $C_{19}H_{26}O_7$ was established. CI mass spectrum contained $[MH-HOAc]^+$ (*m/z* 307), $[MH-HOAc-H_2O]^+$ (*m/z* 289) and $[MH-2HOAc]^+$ (*m/z* 247) ions, implying the existence of a hydroxy group (instead of hydroperoxy in comparison with **1**) and two acetoxy groups in the molecule. The similarity of the ^{1}H NMR data of **2** (Table 1) to that of **1** indicated the same basic structure.^{1}H NMR spectrum of

For Peer Review

2 exhibited signals of two acetoxy groups (δ 2.03 *s* and δ 2.08 *s*, respectively) and their geminal protons at δ 5.29 *dd* (J_1 = 5.0 Hz, J_2 = 11.4 Hz, H-3) and δ 5.10 *brdd* (J_1 = 3.2 Hz, J_2 = 7.6 Hz, H-9). Signals of H-5 (δ 5.39 *brd*, *J* = 10.4 Hz), Me-15 (δ 1.58 *d*, *J* = 1.6 Hz), H-14 (δ 5.56 *brs*) and H-14' (δ 5.48 *brs*) documented double bonds Δ^4 and $\Delta^{10(14)}$, respectively. Broad doublet at δ 4.16 (*J* = 9.8 Hz) originated from H-1, which is geminal to the hydroxy group. Missing the characteristic low-field H-13 and H-13' doublets of the exomethylene group, as well as *brs* of the hydroperoxy group, and the appearance of quintet of H-11 (δ 2.74, *J* = 7.6 Hz) and doublet of Me-13 (δ 1.19, *J* = 7.6 Hz) were the main distinctions of ^{1}H NMR spectrum of **2**, in comparison with **1**. It is very possible that the lactone **2** (3,9-diacetoxy-1-hydroxy-6*β*,7*α*,11H-germacra-4,10(14)-dien-12,6*α*-olide) was formed from sintenin, via corresponding 1,10-epoxy derivative. The hydroperoxide **1** and sintenin might have the same precursor, most probably costunolide or corresponding mono- or di-acetoxy derivative.

Table 1

Molecular formula $C_{18}H_{22}O_7$ for lactone **3** was concluded from HRESI mass spectrum, recorded in the positive-ion mode, using ammonium formiate for the ionisation ($[M+NH_4]^+$ at m/z 368.17165, calc. 368.17038; $[M+Na]^+$ at m/z 373.12752, calc. 373.12577). In EI mass spectrum, ions $[M]^+$ (*m/z* 350), $[MH-HOAc]^+$ (*m/z* 290) and $[MeC=O]^+$ (*m/z* 43) were discerned, which implied the presence of an acetoxy group and the methyl ketone group. The IR spectrum revealed four carbonyls with absorption bands at 1765 cm^{-1} (*γ*-lactone), 1749 cm^{-1} (OAc), 1733 cm^{-1} (C=O) and 1708 cm^{-1} (C=O). In the ^{1}H NMR spectrum (**Table 1**) characteristic doublets for α-methylene-γ-lactone group (H-13: δ 6.40, *J* = 2.6 Hz; H-13': δ 5.67, *J* = 2.0 Hz) and a multiplet

For Peer Review

of H-7 (δ 3.19) were present. These protons, coupled with each other, were a part of one of the two spin systems recognized in TOCSY spectrum (**Fig. 3**). Besides H_2-13 and H-7, this system included H-6 (δ 5.06 *d*, *J* = 4.6 Hz), H_2-8 (δ 2.09 *t*, *J* = 7.0 Hz) and H-9 (δ 5.13 *t*, *J* = 6.8 Hz), a low-field proton geminal to an acetoxy group. A singlet of OAc was partly overlapped (δ 2.15-2.17) with singlets of two methyl groups bound to sp^2-carbons. One of these signals was assigned to Me-14 at carbonyl C-10 and the other to Me-15 at olefinic C-4 (double bound to C-5). The multiplicity of H-6 indicated C-5 to be quarternary. These data, together with the evidence about the existence of separated ABX spin system of five-membered ring, indicated *iso-seco*-guaianolide structure of **3**. ABX system was constituted of diastereotopic CH_2-2 group (A: δ 2.70 *dd* (J_1 = 6.2 Hz, J_2 = 18.4 Hz), B: δ 2.34 *dd* (J_1 = 2.2 Hz, J_2 = 18.4 Hz)) and a proton H-3 (X: δ 4.31 *brd* (*J* = 4.8 Hz)) geminal to OMe group (δ 3.42 *s*). Therefore, **3** is 9-acetoxy-3-*O*-methyl-*iso-seco*-tanapartholide. In NOESY spectrum, more or less expected correlations which confirm the former structure appeared: H_2-8/H-13', H-9, H-6, OAc, H-7; H-2/H-2', H-3, OMe; H-2'/H-2, H-3; H-7/H-13', H-6. Because of the remote nature of the C-3 stereocenter and the flexibility of the (C-8)-(C10) fragment, there was not an evidence about stereochemistry at C-3 and C-9. The configuration at C-9 was deduced on the basis of the previous findings that *seco*-guaianolides, *endo*-peroxyguaianolides and bis-epoxy-guaianolides were biosinteticaly related: all derived from common cyclopentadiene precursors; *seco*- and *iso-seco*-guaianolides formed by a cleavage of (C-1)-(C-10) bond of corresponding epoxy-guaianolides [20]. Accordingly, 9(*R*)-configuration of **3** is proposed, as compatible with 9α-position of acetoxy group of presented endoperoxide **4** and bis-epoxide **5**.

Seco-guaianolides and *iso-seco*-guaianolides mainly occurred in the genera *Artemisia*, *Tanacetum* and *Achillea*. *Iso-seco*-tanapartholide, firstly isolated from *Artemisia rutifolia* in 1986 [21], has

For Peer Review

recently been identified in *Tanacetum parthenium,* together with its 3-epimer (3α-OH), and afterwards both were synthesized. A little difference in NMR data of two epimers and unequal optical rotations were exploited for the comprehensive stereochemical revision of previously isolated '*iso*-*seco*-tanapartholides' from different plants [22]. *Epi*-*iso*-*seco*-tanapartholide was recognized as a major product in some *Achillea* species.

Reviewing literature for 3-epimers of 3-*O*-methyl-*iso*-*seco*-tanapartholides we could not find strong evidence for its distinction [11, 23, 24]. Therefore, we gave up resolving C-3 configuration on the basis of comparing our data with existing spectroscopic data. As well as in the case of *iso*-*seco*-tanapartholides, synthesis of these epimeric compounds is essential for their complete characterization and also required for a revision of the preceding literature data.

Fig. 3

The guianolide **5** was easily determined as 9α-acetoxycanin due to the similarity of its spectral data with those of the previously isolated diastereomer 9α-acetoxyartecanin from the same plant [9]. 9α-Acetoxycanin was also found in *A. depressa* [11]. In the previous study of 1D and 2D NMR data of eleven 1,2-epoxy- and 1,2:3,4-diepoxy-guianolides, which we had isolated from different *Achillea* species, we noted a relation between the chemical shifts of C(H)-5 fragment and the configuration of the 1,2-epoxy ring [25]. Briefly, a signal of H-5 (vicinal to the epoxide) of 1α,2α-epoxyguaianolides is shielded, while C-5 is deshielded in comparison with corresponding data of appropriate β-epoxy-diastereomers. Here, signals of H-5 and C-5 of **5** appeared at δ 2.65 and δ 50.6, respectively, while the signals of its *bis*-β-epoxy-diastereomer 9α-

For Peer Review

acetoxyartecanin were noticed at δ_H 2.87 and δ_C 43.0 [9], which is one more confirmation of the mentioned, previously recognized spectral rule for this type of compounds.

For testing the *in vitro* cytotoxicity of *A. clavennae* sesquiterpenoids, we have selected the predominant metabolite germacranolide sintenin (**6**), lactone apressin (**4**) and the new *iso-seco*-guaianolide (**3**). The glioma cell lines C6 and U251 were chosen as targets, having in mind that gliomas, the most common primary malignancy of the central nervous system, are extremely aggressive and mostly incurable tumors [26]. The treatment with compound **6** for 24 h had no significant influence on either mitochondrial respiration or numbers of glioma cells (Fig. 4), which is consistent with previous results obtained in various cancer cell lines [27, 28]. Compound **4** was toxic to all tested cells, including primary astrocytes (Fig. 4), which is in accordance with the earlier reports [9]. The new *iso-seco*-guaianolide (**3**) also displayed a significant dose-dependent cytotoxicity towards glioma cells, as determined both by MTT assay for mitochondrial respiration and crystal violet test for cell numbers (Fig. 4). The analysis of the IC_{50} values presented in **Table 2** indicates that compound **3** was significantly more active against glioma cells than compound **4**, but somewhat less efficient than the prototypical anticancer drug cisplatin. Importantly, unlike compound **4** and cisplatin, compound **3** displayed only a limited toxicity towards rat primary astrocytes (Fig. 4). While this could be due to a lower proliferation rate of primary astrocytes, it nevertheless indicates that transformed glial cells might be more selective to compound **3** than their primary counterparts.

Fig. 4

Table 2

Georg Thieme Verlag KG, P.O. Box 30 11 20, D-70451 Stuttgart, Germany www.thieme.de/plantamedica

After confirming the *in vitro* antiglioma action of compound **3**, we next assessed its ability to induce programmed cell death known as apoptosis. We first examined activation of caspases, the main apoptosis-executing enzymes [29], using fluorescent-labeled pan-caspase inhibitor ApoStat (Fig. 5A). Flow cytometric analysis revealed that compound **3** induced a dose- dependent caspase activation in both C6 and U251 glioma cells. Furthermore, the cell cycle analysis after PI staining of cellular DNA demonstrated that the exposure to compound **3** increased the numbers of hypodiploid glioma cells with fragmented DNA (sub-G0/G1 compartment) (Fig. 5B), which is a hallmark of apoptosis [30]. Annexin-detectable externalization of phosphatidylserine is another apoptotic marker [31], and the treatment with compound **3** increased the numbers of annexin$^+$ glioma cells displaying phosphatidylserine in the outer leaflet of cell membrane (Fig. 5C). The effects of compound **3** (25 µM) on DNA fragmentation (21.5% hypodiploid cells) and phosphatidylserine exposure (32.8% annexin$^+$ cells) in U251 cells were only slightly less prominent than those of cisplatin at the same concentration (32.4% and 37.8%, respectively; data not shown). These results clearly demonstrate that compound **3** induces apoptotic death of glioma cells. However, it should be noted that compound **3** also increased the proportion of PI$^+$ cells with the cell membrane damage (Fig. 5C), which could be late apoptotic and/or necrotic cells. Therefore, the possibility that compound **3** could actually induce mixed apoptotic/necrotic death requires further investigation.

Fig. 5

Oxidative-stress mediated mitochondrial dysfunction can lead to activation of caspases and subsequent apoptotic cell demise [31]. Thus, we next examined the effects of compound **3** on

For Peer Review

ROS production and mitochondrial membrane potential in glioma cells. The flow cytometric analysis of cells stained with the redox-sensitive dye DHR demonstrated that compound **3** was able to induce a dose- dependent ROS generation in glioma cell lines (Fig. 6A). The induction of oxidative stress in compound **3**-treated C6 glioma cells was associated with the mitochondrial membrane depolarization, as confirmed by the increase in green-to-red fluorescence ratio (FL1/FL2) of the mitochondria-binding dye DePsipher (Fig. 6B). While these data indicate that oxidative stress and subsequent mitochondrial depolarization are involved in the cytotoxicity of compound **3**, this assumption remains to be directly confirmed.

Fig. 6

The previously isolated structurally related *iso-seco*-guaianolides were found to reduce viability of immortalized human keratinocytes [32] and inhibit nitric oxide synthesis in lipopolysaccharide-activated RAW 264.7 cells [33], probably through interaction with NF-κB signaling pathway [22]. The high biological activities of these compounds, including new *iso-seco*-guaianolide **3**, are most likely connected with their α,β-unsaturated carbonyl electrophilic groups: an α-methylene-γ-lactone and an α,β-unsaturated cyclopentenone. These groups are known to mediate a Michael-type addition reaction with thiol-containing enzymes and proteins. The importance of these structural features is primarily supported by the fact that lactone **6** (without mentioned groups) was inactive in the present study. Accordingly, lactone **4** with only one α,β-unsaturated carbonyl group was less active than **3**.

Taken together, our data for the first time demonstrate that compound **3**, a novel *iso-seco*-tanapartholide from *A. clavennae*, triggers apoptotic death of glioma cells associated with the induction of oxidative stress and mitochondrial depolarization.

For Peer Review

Materials and methods

General experimental procedures

Optical rotations were determined on a Rudolph Research Analytical automatic polarimeter, Autopol IV. IR spectra were measured in the form of transparent films on a Perkin-Elmer FT-IR spectrometer 1725X. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (at 200 and 50 MHz, respectively) and an Bruker Avance III spectrometer (at 500 and 125 MHz, respectively) in $CDCl_3$, using TMS as internal standard. Mass spectra were obtained on a Finnigan MAT 8230, BE DCI (150 eV, iso-butane). High-resolution LC/ESI TOF mass spectra were measured on a HPLC instrument (Agilent 1200 Series) with a Zorbax Eclipse Plus C18 column (150 x 4.6 mm i.d.; 1.8 µm) and a diode-array detector (DAD) coupled with a 6210 time-of-flight LC/MS system (Agilent Technologies). Elemental analysis was performed on a Vario EL III C,H,N,S/O Elemental Analyzer (Elementar). CC was carried out on silica gel 60 (0.063-0.200 mm, Merck). Preparative TLC was performed on silika gel G and F_{254} (0.75 mm layer, 20 × 20 cm). Silica gel 60 F_{254} precoated aluminum sheets (0.25 mm, Merck) were used for TLC control. Spots were detected under UV254 and by spraying with 50% H_2SO_4.

Plant material

The aerial parts of *Achillea clavennae* L. (Asteraceae) were collected at the flowering stage in July 2002 in Montenegro at the mountain Prokletije (Karanfili, altitude ca. 2000 m). The plant sample was identified by Prof. Pedja Janaćković (Faculty of Biology, University of Belgrade,

For Peer Review

Belgrade, Serbia), and a voucher specimen (BEOU16677) was deposited in the BEOU-Herbarium, Institute of Botany, Faculty of Biology (Belgrade, Serbia).

Extraction and isolation

The air-dried aerial parts of *A. clavennae* (270 g) were extracted at a room temperature with a mixture of petrol-Et_2O-MeOH (1:1:1, 2.1 L, 24 h) using the standard procedure [34]. The initial extract (9.0 g) was treated with MeOH (3 × 50 mL) to yield 7.2 g of MeOH soluble portion. Further fractionation was carried out on silica gel dry-column flash chromatography (250 g, 10 × 14 cm), starting the elution with petrol (fraction **F1**, 400 mL) and increasing polarity by adding Et_2O (20% to 100%, **F2-F10**, 350 mL each) and MeOH (15%, **F11**, 350 mL). Fractions were evaporated, and **F7** (petrol-Et_2O 3:7, 423 mg) and **F8** (petrol-Et_2O 2:8, 441 mg) were combined after inspection of analytical TLC (CH_2Cl_2-MeOH 97:3) and ^{1}H NMR spectra of all fractions.

F6 (petrol-Et_2O 4:6, 558 mg) was chromatographed on silica gel CC (300 g, 45 × 3.5 cm; CH_2Cl_2-MeOH 97:3) to yield 48 subfractions (~20 mL each). Fractions 7-12 (F6a, 62.4 mg), 22 (F6b, 24.1 mg), and 25-29 (F6c, 51.0 mg) were further purified by preparative TLC. Sesamin (3.2 mg, F6a/CH_2Cl_2-MeOH 97.5:2.5), apressin (**4**, 3.5 mg, F6b/CH_2Cl_2-MeOH 97:3) and oleanolic acid (**7**, 2.1 mg, F6c/CH_2Cl_2-MeOH 97.5:2.5, repeated prep.TLC) were obtained.

F7+8 (864 mg) was divided into 75 subfractions (~30 mL each) by silica gel CC (430 g, 70 × 3.5 cm) gradually increasing polarity of eluent (CH_2Cl_2-MeOH, 97.4:2.6 - 96:4). Subfr. F7a (23-26, 36.6 mg), F7b (27-33, 74.2 mg), F7c (34-49, 102.1 mg), F7d (54-57, 32.3 mg), and F7e (71-75, 56.2 mg) were purified by preparative TLC (always at several plates). Lactone **2** (1.1 mg) was isolated from F7a and lactone apresin (**4**, 10.9 mg) from F7b with the same solvent system (CH_2Cl_2-MeOH 96.5:3.5). Lactone **3** (5.4 mg) was obtained from F7c (CH_2Cl_2-MeOH 97:3). 11-Dehydroxy,11-hydroperoxyindicumenone (1.4 mg) was obtained from F7d together with

For Peer Review

additional 1.1 mg of **3** (CH_2Cl_2-MeOH 96.5:2.5, repeated TLC). Chrysetunone (0.5 mg) and centaureidin (1.4 mg) were isolated from F7e (CH_2Cl_2-MeOH 97:3).

Silica gel CC (300 g, 45 × 3.5 cm; CH_2Cl_2-MeOH 97:3) of **F9** (petrol-Et_2O 1:9, 495 mg) yielded 40 subfractions (~30 mL each), which were combined after analytical TLC. F9a (4-5, 37.5 mg) yielded lactones **4** (8.5 mg), **1** (1.4 mg), and **6** (1.0 mg) after repeated preparative TLC (solvent system CH_2Cl_2-MeOH 97:3). F9b (6-7, 28.2 mg) was washed out with Et_2O and an insoluble portion was crystallized from CH_2Cl_2 giving 11.4 mg of sintenin (**6**). 9α-Acetoxycanin (**5**, 0.9 mg) was isolated from F9c (13-16, 33.1 mg) after repeated preparative TLC with solvent systems toluen-Et_2O-MeOH (7:2:1) and CH_2Cl_2-MeOH (97:3).

Lactone sintenin (10.0 mg) was also isolated from **F10** (Et_2O, 450 mg), which was firstly divided into 20 subfractions (~30 mL each) by silica gel CC (280 g, 40 × 3.5 cm) using solvent system CH_2Cl_2-MeOH (97:3). From combined fraction 4-8 (65.3 mg), this compound was separated as insoluble in Et_2O.

3β,9β-diacetoxy-1β-hydroperoxy-6β,7αH-germacra-4,10(14),11(13)-trien-12,6α-olide (**1**): Yellowish gum; $[\alpha]_D^{22}$ +30.2 (CH_2Cl_2, *c* 0.119); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 383 $[MH]^+$ (~21), 351 $[MH\text{-}O_2]^+$ (~34), 291 $[351\text{-}HOAc]^+$ (~5), 263 $[MH\text{-}2HOAc]^+$ (~63), 245 $[263\text{-}H_2O]^+$ (~68); HRESIMS: *m/z* 381.15398 $[M+H]^+$ (calculated for $C_{19}H_{24}O_8$+H 381.15439); ^1H and ^{13}C NMR data, see **Table 1**; COSY and NOESY data, see **Fig. 2**; HMBC: H_3-15/C-3, C-4, C-5; H_2-14/C-9; H-14/C-10; H-13/C-7, C-11; H-3/C-5.

3,9-diacetoxy-1-hydroxy-6β,7α,11H-germacra-4,10(14)-dien-12,6α-olide (**2**): Yellow gum; $[\alpha]_D^{22}$ +24.0 (CH_2Cl_2, *c* 0.101); CIMS (iso-butane, probe), 150 eV, *m/z* (rel. int.): 367 $[MH]^+$

For Peer Review

(~58), 307 $[MH\text{-}HOAc]^+$ (~82), 289 $[307\text{-}H_2O]^+$ (~54), 247 $[307\text{-}HOAc]^+$ (~33); HRESIMS: *m/z* 367.17513 $[M+H]^+$ (calculated for $C_{19}H_{26}O_7$+H 367.17485); ^{1}H NMR data, see **Table 1**.

9(R)-acetoxy-3-O-methyl-iso-seco-tanapartholide (**3**): White crystals; $[\alpha]_D^{22}$ –10.7 (CH_2Cl_2, *c* 0.168); IR ν_{max}^{film} (cm^{-1}): 1765 (γ-lactone), 1749 (OAc), 1733 (C=O), 1708 (C=O); ^{1}H and ^{13}C NMR data, see **Table 1**; HMBC: H_2-8/C-9, C-11, O<u>C</u>OCH_3; OMe/C-3; H-13/C-11; TOCSY data, see **Fig. 3**; NOESY: H_2-8/H-13', H-9, H-6, OAc, H-7; H-2/H-2', H-3, OMe; H-2'/H-2, H-3, OMe; H-7/H-13', H-6; EIMS (70 eV), *m/z*: 350 $[M]^+$, 290 $[M\text{-}HOAc]^+$, 247 $[290\text{-}COCH_3]^+$, 215 $[247\text{-}HOCH_3]^+$; HRESIMS: *m/z* 368.17165 $[M+NH_4]^+$ (calculated for $C_{18}H_{22}O_7$+NH_4 368.17038) and *m/z* 373.12752 $[M+Na]^+$ (calculated for $C_{18}H_{22}O_7$+Na 373.12577); Elemental analysis: found C - 61.60, H - 6.42; requires: C - 61.71, H - 6.33%.

Cells and reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Human glioma cell line U251 and rat glioma cell line C6 were kindly donated by Dr Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The primary astrocytes were isolated from brains of newborn Albino Oxford rats as previously described [35], in accordance with the ethical guidelines stated in the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). Tumor cell lines and primary rat astrocytes were grown in HEPES (20 mM)-buffered RPMI 1640 cell culture medium, supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 10 mM sodium pyruvate and 1% of antibiotic/antimycotic mixture at 37 °C in a humidified atmosphere with 5% CO_2. The cells were prepared for experiments using trypsin/EDTA and incubated in 96-well flat-bottom cell culture plates (2×10^4 cells/well) for the cell viability

For Peer Review

assessment, or 6-well plates (5×10^5 cells/well) for the flow cytometric analysis (plates were from Sarstedt, Nümbrecht, Germany). Cells were rested for 24 h and then treated with compounds **3, 4** and **6**. Purity of **3, 4** and **6** was confirmed by elemental analysis (> 99%). Control cultures were exposed to the corresponding amount of vehicle (DMSO). We observed no influence of DMSO on any of the parameters tested (data not shown). Cisplatin (≥ 99.9%, Sigma-Aldrich), a well known anticancer drug was used in some experiments as a positive control. Incubation times and concentrations of compounds are stated in figure legends.

Cell viability

Mitochondrial dehydrogenase activity, as an indicator of cell viability, was assessed by mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, and the number of viable cells was determined using crystal violet assay, exactly as previously described [36]. The MTT and crystal violet absorbance was measured in an automated plate reader (Sunrise, Tecan, Dorstet, UK) at 570 nm. The IC_{50} values were calculated using GraphPad Prism software.

Caspase activation

Activation of caspases was measured using cell-permeable FITC-conjugated pan-caspase inhibitor ApoStat (R&D Systems, Minneapolis, MN), according to manufacturer's instructions. The increase in green fluorescence (FL1) as a measure of caspase activity was detected using FASCalibur flow cytometer (BD, Heidelberg, Germany). The results are presented as mean intensity of ApoStat fluorescence.

Apoptosis analysis

For Peer Review

DNA fragmentation as a marker of apoptotic cell death was analyzed by measuring the DNA content of ethanol-fixed cells stained with DNA-binding dye propidium iodide (PI), exactly as previously described [36]. Red (FL2) fluorescence of PI-stained cells was measured, and percentage of hypodiploid, apoptotic cells (sub-G compartment of the cell cycle) was determined. Alternatively, apoptotic cell death was assessed after double staining with annexinV-FITC and PI, according to manufacturer's instructions (BD, Heidelberg, Germany). Annexin binds the phosphatidylserine exposed on the membrane of apoptotic cells, while PI labels the DNA in cells with membrane damage. The green (FL1) and red (FL2) fluorescence of annexinV-FITC/PI labeled cells was analyzed, and annexin$^+$/PI$^-$ and annexin$^+$/PI$^+$ cells were considered as early and late apoptotic cells, respectively. Flow cytometry analyses were conducted on FACSCalibur flow cytometer using CellQuest Pro software (BD, Heidelberg, Germany).

Determination of oxidative stress and mitochondrial membrane potential

Intracellular production of reactive oxygen species (ROS) was determined using redox-sensitive fluorescent dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK). DHR (5 µM) was added to cell cultures at the beginning of the treatment. The mean intensity of green (FL1) fluorescence corresponding to oxidative stress was determined using FACSCalibur flow cytometer. Mitochondrial membrane potential was determined using DePsipher (R&D Systems), a lipophilic cation that has the property of aggregating upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed the dye remains or reverts to its green monomeric form. The cells were stained with DePsipher as described by the manufacturer and fluorescence was detected by flow cytometry. The results are presented as green/red fluorescence ratio (mean FL1/FL2), the increase of which reflects mitochondrial depolarization.

For Peer Review

Acknowledgements

This study was financially supported by the Serbian Ministry of Education, Science and Technological Development (Grant No. 172053 and 41025).

Conflict of Interest

There are no conflicts of interest.

For Peer Review

References

1. *Gajić M*. Genus *Achillea* L. In: Josifović VM, editor. Flora of Serbia VII. Belgrade: Serbian Academy of Sciences and Arts; 1975: 90-110
2. *Trifunović SS.* Comparative investigation of the chemical composition of some plant species of the genus *Achillea* L. [dissertation]. University of Belgrade: Faculty of Chemistry; 2006
3. *Sia XT, Zhanga ML, Shi QW, Kiyota H.* Chemical constituents of the plants in the genus *Achillea*. Chem Biodivers 2006; 3: 1163-1180
4. *Valant-Vetschera KM, Wollenweber E.* Exudate flavonoid aglycones in alpine species of *Achillea* sect. *Ptarmica*. Biochem Syst Ecol 2001; 29: 149-159
5. *Maggioni G.* Nicolò Chiavenna, Bellunese druggist of the sixteenth century and the contested discovery of umbelliferous absinthium (*Achilea clavenae* L.). Minerva Farm 1953; 2: 267-269
6. *Skočibušić M, Bežić N, Dunkić V, Radonić A*. Antibacterial activity of *Achillea clavennae* essential oil against respiratory tract pathogens. Fitoterapia 2004; 75: 733-736
7. *Bežić N, Skočibušić M, Dunkić V, Radonić A.* Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. Phytother Res 2003; 17: 1037-1040
8. *Stojanović G, Radulović N, Hashimoto T, Palić R.* In vitro antimicrobial activity of extracts of four *Achillea* species: the composition of *Achillea clavennae L.* (Asteraceae) extract. J Ethnopharmacol 2005; 101: 185-190
9. *Trifunović S, Vajs V, Juranić Z, Žižak Ž, Tešević V, Macura S, Milosavljević S*. Cytotoxic constituents of *Achillea clavennae* from Montenegro. Phytochemistry 2006; 67: 887-893
10. *Tsankova E, Kempe UJ, Norin T, Ognyanov I.* Apressin, a guaianolide of the endoperoxide type from *Achillea depressa*. Phytochemistry 1981; 20: 1436-1438
11. *Todorova M, Mustakerova E, Tsankova E.* Sesquiterpene lactones from *Achillea depressa* Janka. Dokladi na Bulgarskata Akademiya na Naukite 2005; 58: 25-32

For Peer Review

12. *Goren N, Oksuz S, Ulubelen A.* A Sesquiterpene Lactone, Sintenin from *Achillea sintenisii.* Phytochemistry 1988; 27: 2346-2347

13. *Mladenova K, Tsankova E, van Hung D.* New sesquiterpenoids from *Chrysanthemum indicum* var. *tuneful*. Planta Med 1988; 54: 553-555

14. *Meselhy MR.* Constituents from Moghat, the Roots of *Glossostemon bruguieri* (Desf.). Molecules 2003; 8: 614-621

15. *Long C, Sauleau P, David B, Lavaud C, Cassabois V, Ausseil F, Massiot G.* Bioactive flavonoids of Tanacetum parthenium revisited. Phytochemistry 2003; 64: 567-569

16. *Seebacher W, Simić N, Weis R, Saf R, Kunert O.* Complete assignments of ^{1}H and ^{13}C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives. Magn Reson Chem 2003; 41: 636-638

17. *Oksuz S, Ulubelen A, Tuzlaci E.* Constituents of *Achillea teretifolia*. Fitoterapia 1990; 61: 283

18. *Barrero AF, Alvarez-Manzaneda REJ, Alvarez-Manzaneda RR.* Bisabolene derivatives and other constituents from *Achillea odorata.* Phytochemistry 1990; 29: 3213-3216

19. *Bruno M, Bondi ML, Paternostro MP, Arnold NA, Diaz JG, Herz W.* Bisabolenes from *Achillea cretica.* Phytochemistry 1996; 42: 737-740

20. *Hewlett MJ, Begley MJ, Groenewegen WA, Heptinstall S, Knight DW, May J, Salan U, Toplis D.* Sesquiterpene lactones from feverfew, *Tanacetum parthenium*: isolation, structural revision, activity against human blood platelet function and implications for migraine therapy. J Chem Soc Perkin Trans I. 1996; 16: 1979–1986

21. *Huneck S, Zdero C, Bohlmann F.* Seco-guaianolides and other constituents from Artemisia species. Phytochemistry 1986; 25: 883-889

For Peer Review

22. *Makiyi EF, Frade RFM, Lebl T, Jaffray EG, Cobb SE, Harvey AL, Slawin AMZ, Hay RT, Westwood NJ.* Iso-*seco*-tanapartholides: Isolation, Synthesis and Biological Evaluation. Eur. J. Org. Chem. 2009; 33: 5711-5715

23. *Tan RX, Jia ZJ, Jakupovic J, Bohlmann F, Huneck S.* Sesquiterpene lactones from *Artemisia rutifolia*. Phytochemistry 1991; 30: 3033-3035

24. *Zan K, Chen X-Q, Fu Q, Shi S-P, Zhou S-X, Xiao M-T, Tu P-F.* 10-Secoguaianolides from *Artemisia anomala* (Asteraceae). Biochemical Systematics and Ecology 2010; 38: 431–434

25. *Trifunović S, Milosavljević S, Vajs V, Macura S, Todorović N.* Stereochemistry and conformations of natural 1,2-epoxy-guaianolides based on 1D and 2D NMR data and semiempirical calculations. Magn Reson Chem 2008; 46: 427-431

26. *Giese A, Bjerkvig R, Berens ME, Westphal M.* Cost of migration: invasion of malignant gliomas and implications for treatment. J Clin Oncol 2003; 21: 1624-1636

27. *Csupor-Löffler B, Hajdú Z, Zupkó I, Réthy B, Falkay G, Forgo P, Hohmann J.* Antiproliferative effect of flavonoids and sesquiterpenoids from *Achillea millefolium* s.l. on cultured human tumour cell lines. Phytother Res 2009; 23: 672-676

28. *Hu LH, Zou HB, Gong JX, Li HB, Yang LX, Cheng W, Zhou CX, Bai H, Guéritte F, Zhao Y.* Synthesis and biological evaluation of a natural ester sintenin and its synthetic analogues. J Nat Prod 2005; 68: 342-348

29. *Sadowski-Debbing K, Coy JF, Mier W, Hug H, Los M.* Caspases--their role in apoptosis and other physiological processes as revealed by knock-out studies. Arch Immunol Ther Exp (Warsz) 2002; 50: 19-34

30. *Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P.* Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods 2008; 44: 205-221

For Peer Review

31. *Chandra J, Samali A, Orrenius S.* Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000; 29: 323-33

32. *Ghantous A, Nasser N, Saab I, Darwiche N, Saliba NA.* Structure–activity relationship of seco-tanapartholides isolated from *Achillea falcata* for inhibition of HaCaT cell growth. Eur J Med Chem 2009; 44: 3794–3797

33. *Ahn H, Kim JY, Lee HJ, Kim YK, Ryu J-H.* Inhibitors of inducible nitric oxide synthase expression from *Artemisia iwayomogi.* Arch Pharm Res 2003; 26: 301-305

34. *Bohlmann F, Zdero C, King HR, Robinson EH.* Pseudoguaianolides and other sesquiterpene lactones from Gaillardia species. Phytochemistry 1984; 23: 1979-1988

35. *Trajkovic V, Vuckovic O, Stosic-Grujicic S, Miljkovic D, Popadic D, Markovic M, Bumbasirevic V, Backovic A, Cvetkovic I, Harhaji L, Ramic Z, Mostarica Stojkovic M.* Astrocyte-induced regulatory T cells mitigate CNS autoimmunity. Glia. 2004; 47: 168-179

36. *Kaludjerovic GN, Miljkovic D, Momcilovic M, Djinovic VM, Mostarica Stojkovic M, Sabo TJ, Trajkovic V.* Novel platinum(IV) complexes induce rapid tumor cell death in vitro. Int J Cancer 2005; 116: 479-486

For Peer Review

Figure legends

Fig. 1 The structures of compounds **1**–**7**.

Fig. 2 COSY (a: ⟷) and NOESY (b: ⟷) correlations of **1**.

Fig. 3 TOCSY correlations (⟷) of **3**.

Fig. 4. Antiglioma effects of compounds **3, 4** and **6**. C6 cells, U251 cells and rat primary astrocytes were incubated with different concentrations of compounds **3, 4, 6**, or cisplatin, and mitochondrial respiration (MTT) and cell numbers (crystal violet) were assessed after 24 h (the dashed lines represents the viability of untreated cells, which was arbitrarily set to 100%). The results are presented as mean ± SD values from three independent experiments.

Fig. 5 Compound **3** induces apoptosis in glioma cells. (A-C) U251 cells (left) and C6 cells (right) were incubated with 12.5 µM (A) or 25 µM (A-C) of compound **3** for 24 h. Cells were stained with pan-caspase inhibitor ApoStat (A), PI (B) or annexinV-FITC/PI (C), and caspase activity (A), DNA fragmentation (B) and phoshatidyl-serine exposure (C) were assessed using flow cytometry. The histograms (A, B) and dot-plots (C) from a representative of three independent experiments are presented.

Fig. 6 Compound **3** induces oxidative stress and mitochondrial depolarization in glioma cells. (A) U251 and C6 cells were incubated with compound **3** (12.5 µM or 25 µM) for 24 h and ROS production in DHR-stained cells was investigated using flow cytometry. The histograms from a

For Peer Review

representative of three independent experiments are shown. (B) C6 cells were incubated with compound **3** (25 µM) for 24 h and mitochondrial membrane potential in DePsipher-stained cells was assessed using flow cytometry. The histograms from a representative of three independent experiments are presented (the values of FL1/FL2 are mean fluorescence ratios reflecting mitochondrial depolarization).

For Peer Review

Table 1 NMR data of **1**, **3** (500 MHz for ^{1}H, $CDCl_3$) and **2** (200 MHz for ^{1}H)

H/C	**1**		**2**	**3**	
	δ_H	δ_C[a]	δ_H	δ_H	δ_C[a]
1	4.24 *brd* (10.0)	86.1	4.16 *brd* (9.8)		c
2α	2.50 *dddd* (1.5, 5.0, 13.0)	34.8	2.55 *ddd* (1.5, 5.0, 12.8)	2.70 *dd* (6.2, 18.4),	40.7
2β	2.11 *m*[b]		2.05 *m*[b]	2.34 *dd* (2.2, 18.4)	
3	5.33 *dd* (5.0, 11.5)	75.6	5.29 *dd* (5.0, 11.4)	4.31 *brd* (4.8)	79.8
4		144.2			173.1
5	5.45 *brd* (10.5)	122.2	5.39 *brd* (10.4)		c
6	4.31 *dd* (9.5, 10.0)	79.0	4.33 *t* (10.0)	5.06 *brd* (4.6)	77.0
7	2.84 *ddd* (2.0, 10.0, 10.0)	46.8	2.05 *m*[b]	3.19 *m*	40.5
8α	2.29 *brd* (14.5)	33.2	2.45 *m*	2.09 *t* (7.0)	34.2
8β	1.93 *brdd* (14.5, 10.0)		2.05 *m*[b]		
9	5.14 *brd* (10.5)	76.8	5.10 *brdd* (3.2, 7.6)	5.13 *t* (6.8)	75.5
10		145.3			c
11		138.0	2.74 *quint* (7.6)		138.0
12		168.8			c
13	6.28 *d* (3.5)	119.8	1.19 *d* (7.6)	6.40 *d* (2.6)	124.0
13’	5.52 *d* (3.0)			5.67 *d* (2.0)	
14	5.56 *brs*	117.6	5.56 *brs*	2.16 *s*[b]	29.6
14’	5.46 *brs*		5.48 *brs*		
15	1.58 *brs*	12.6	1.58 *d* (1.6)	2.16 *s*[b]	14.3
OOH	8.75 *brs*				
OAc	2.08 *s*[b]	170.2, 20.7	2.08 *s*	2.17 *s*	171.2, 20.6
OAc	2.06 *s*[b]	169.4, 21.0	2.03 *s*	/	/
OMe	/	/	/	3.42 *s*	57.6

Georg Thieme Verlag KG, P.O. Box 30 11 20, D-70451 Stuttgart, Germany www.thieme.de/plantamedica

For Peer Review

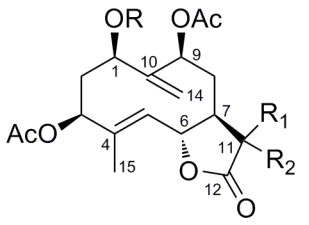
[a] Detected via HSQC and HMBC spectra. [b] Overlapped signals. [c] Not observed.

Table 2. IC_{50} values for compounds **3**, **4**, **6**, and cisplatin, presented as mean ± SD from three independent experiments (*$p < 0.05$ compared to **4**, t-test; CV - crystal violet, n.a. - not assessed).

	comp. 6		**comp. 4**		**comp. 3**		**cisplatin**	
	MTT	CV	MTT	CV	MTT	CV	MTT	CV
U251	>100	>100	91.8±7.9	97.7 ± 9.4	36.6 ± 6.8*	50.9 ± 7.2*	20.8 ± 4.3	18.9 ± 3.2
C6	>100	>100	80.1 ± 9.6	90.0 ± 10.5	41.6 ± 8.5*	66.6 ± 9.5*	15.0 ± 5.7	17.6 ± 4.1
astrocytes	n.a.	n.a.	>100	61.8 ± 15.6	>100	>100	42.1 ± 9.1	25.5 ± 5.1

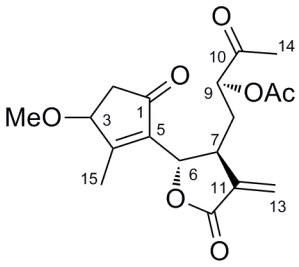
For Peer Review

Fig. 1

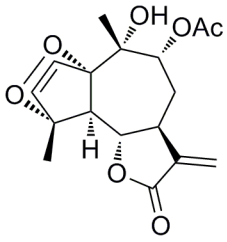


1 R=OH, R_1,R_2=CH_2

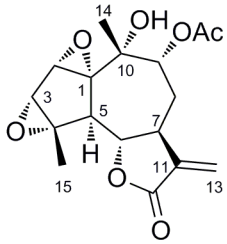
2 R=H, R_1=Me, R_2=H



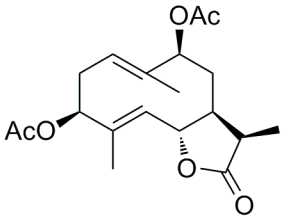
3



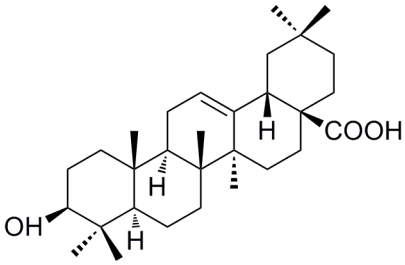
4



5



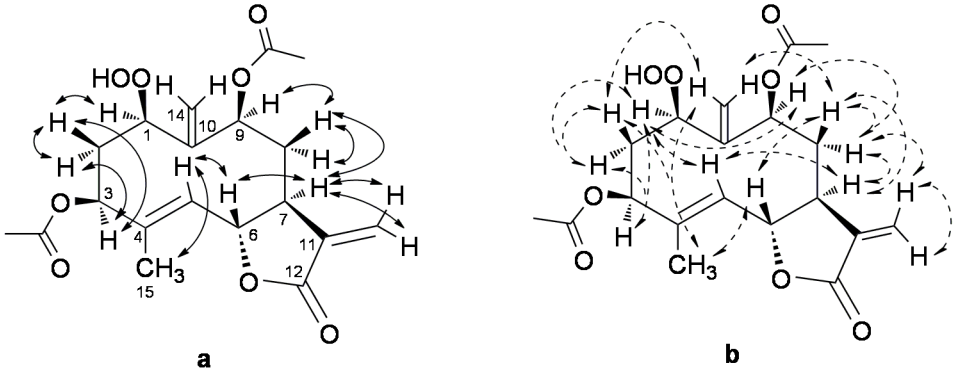
6



7

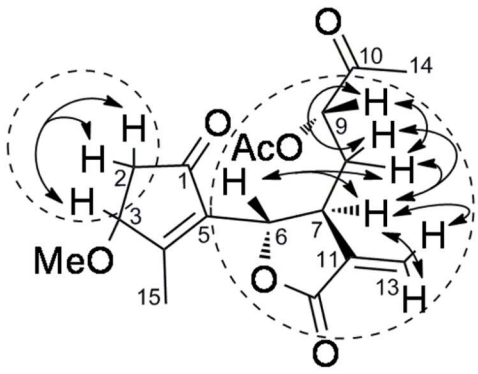
er Review

Fig. 2



For Peer Review

Fig. 3



For Peer Review

Fig. 4

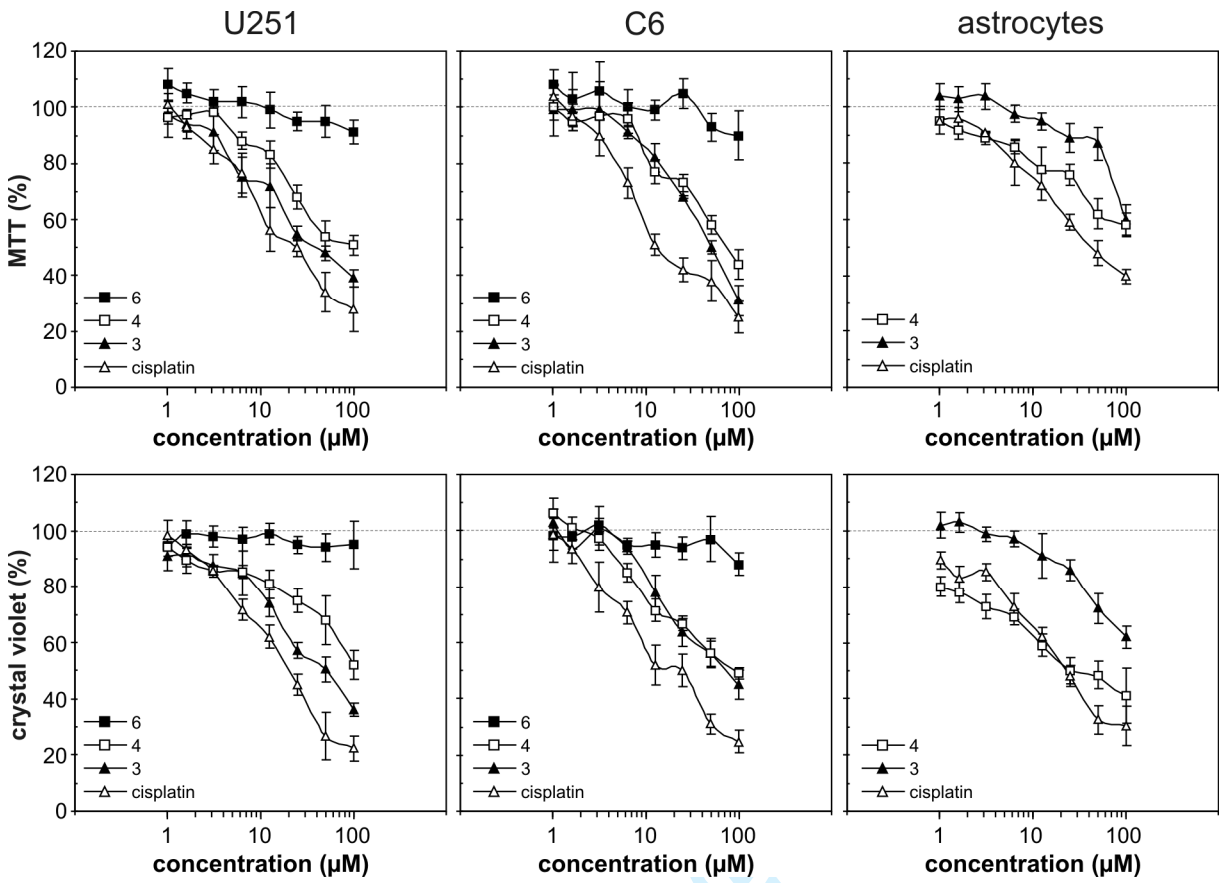

U251
C6
astrocytes
MTT (%)
crystal violet (%)
concentration (μM)
6
4
3
cisplatin

Fig. 5

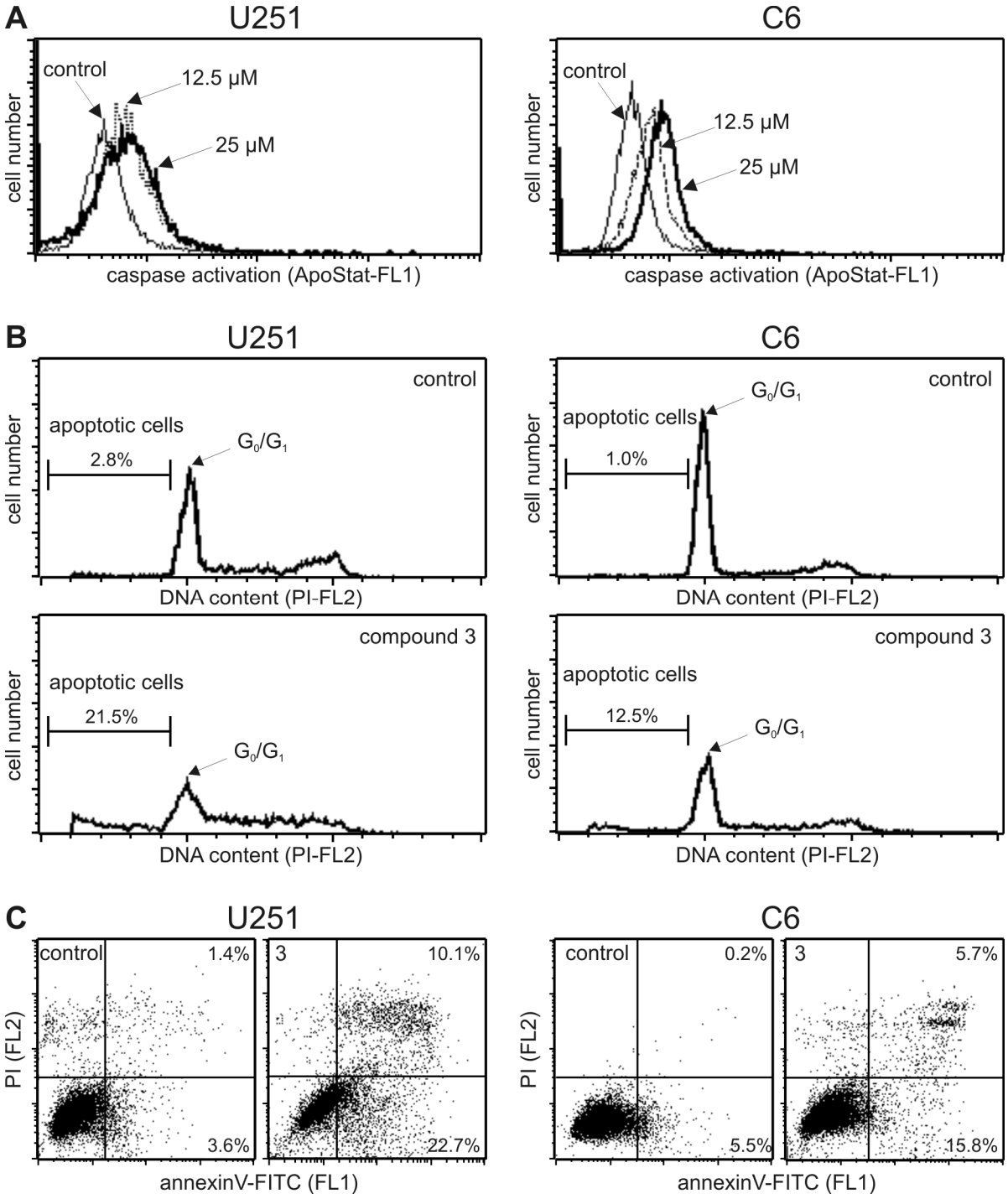
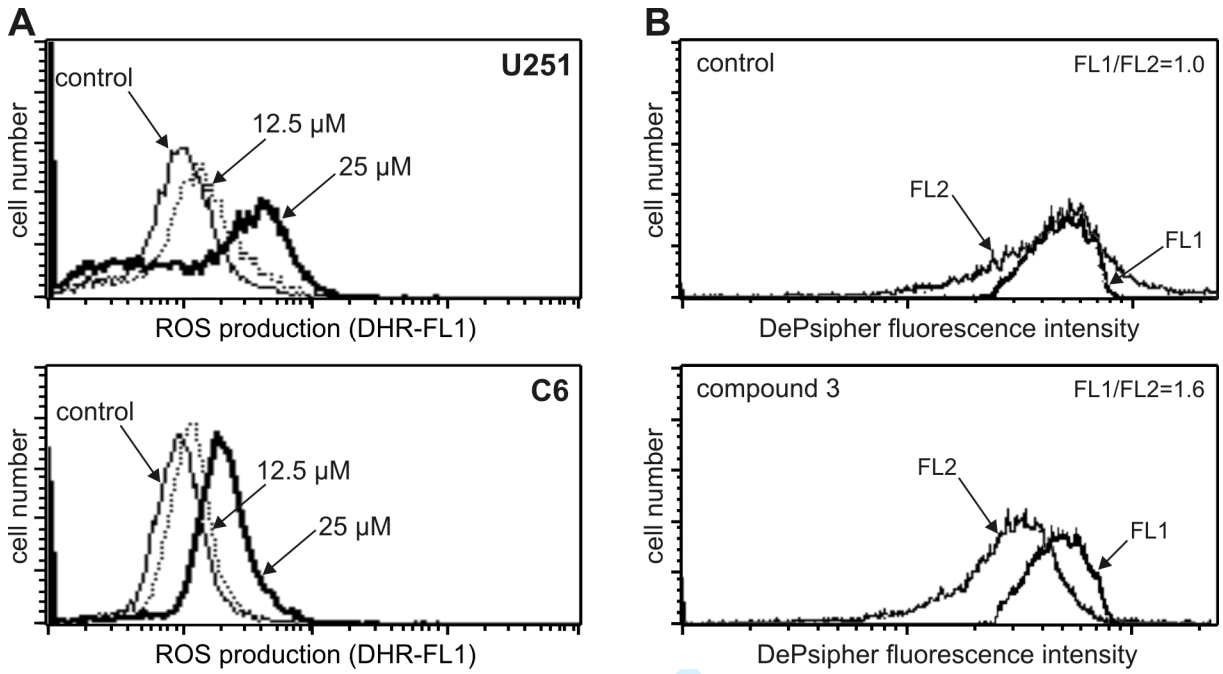
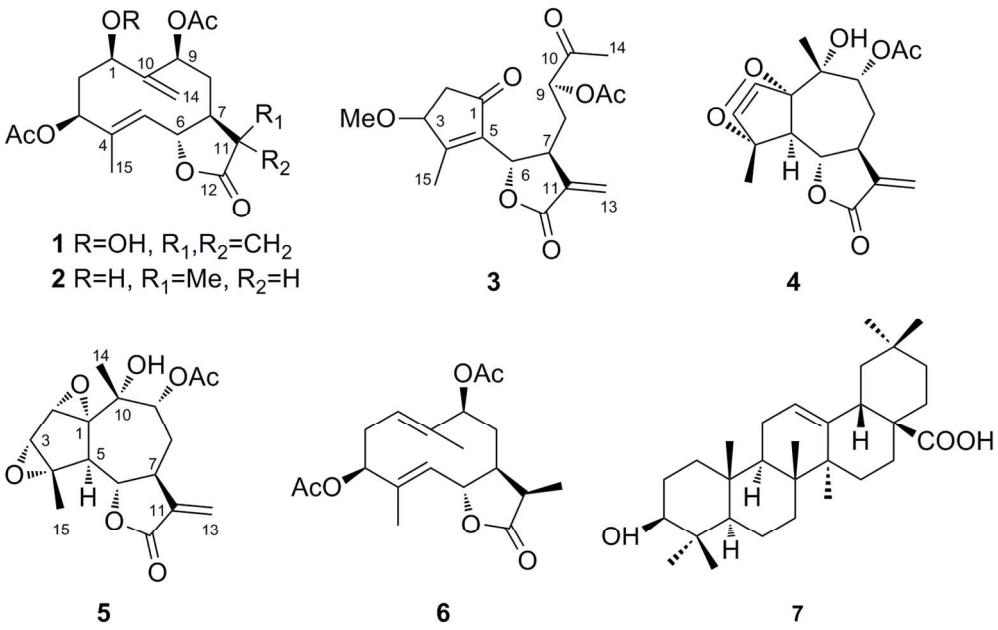

A
U251
control
12.5 µM
25 µM
cell number
caspase activation (ApoStat-FL1)
C6
control
12.5 µM
25 µM
cell number
caspase activation (ApoStat-FL1)
B
U251
control
apoptotic cells
2.8%
G0/G1
cell number
DNA content (PI-FL2)
C6
control
G0/G1
apoptotic cells
1.0%
cell number
DNA content (PI-FL2)
compound 3
apoptotic cells
21.5%
G0/G1
cell number
DNA content (PI-FL2)
compound 3
apoptotic cells
12.5%
G0/G1
cell number
DNA content (PI-FL2)
C
U251
control
1.4%
3.6%
3
10.1%
22.7%
PI (FL2)
annexinV-FITC (FL1)
C6
control
0.2%
5.5%
3
5.7%
15.8%
PI (FL2)
annexinV-FITC (FL1)

Fig. 6


A
U251
control
12.5 µM
25 µM
cell number
ROS production (DHR-FL1)
C6
control
12.5 µM
25 µM
cell number
ROS production (DHR-FL1)
B
control
FL1/FL2=1.0
FL2
FL1
cell number
DePsipher fluorescence intensity
compound 3
FL1/FL2=1.6
FL2
FL1
cell number
DePsipher fluorescence intensity

Review



134x84mm (300 x 300 DPI)

For Review

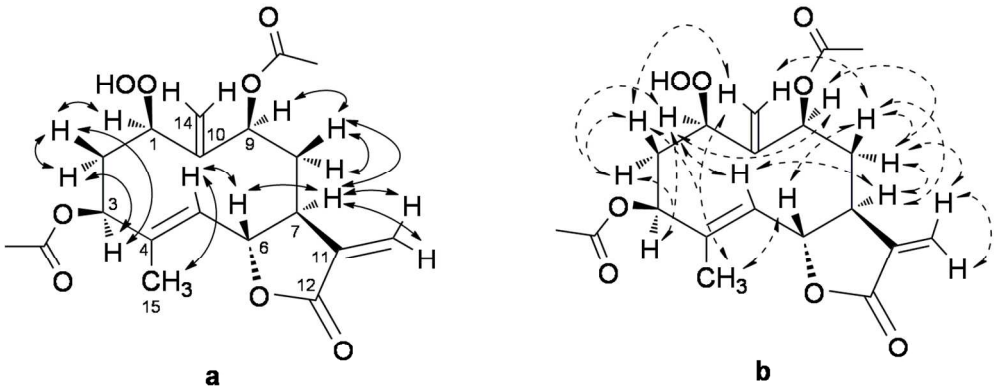


Fig. 2
126x54mm (300 x 300 DPI)

Peer Review

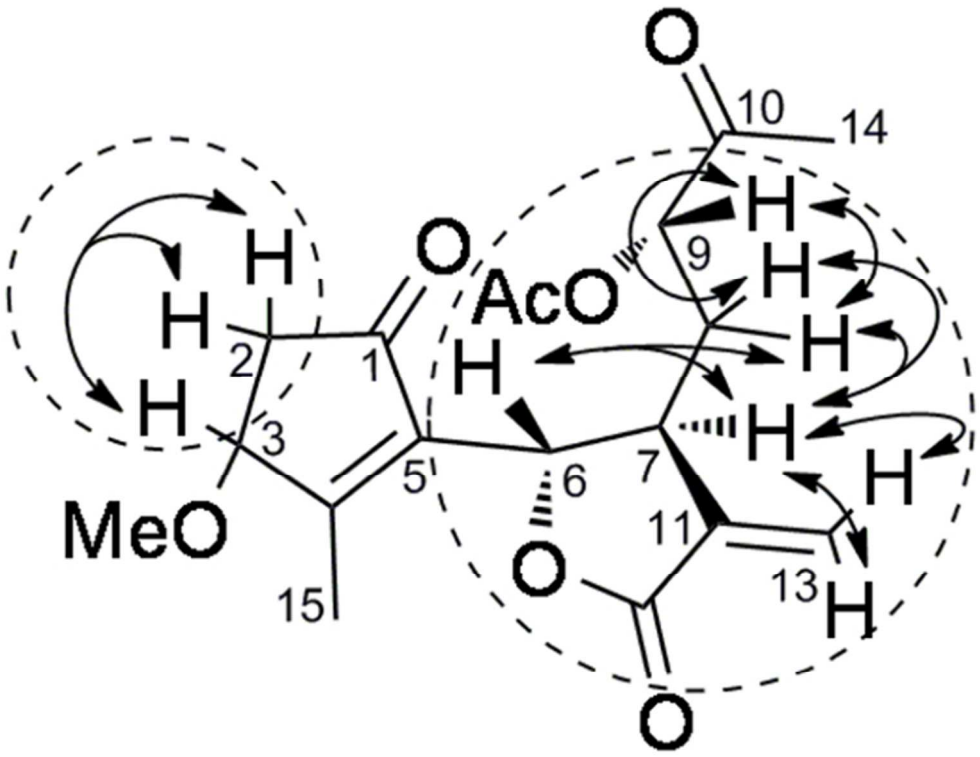
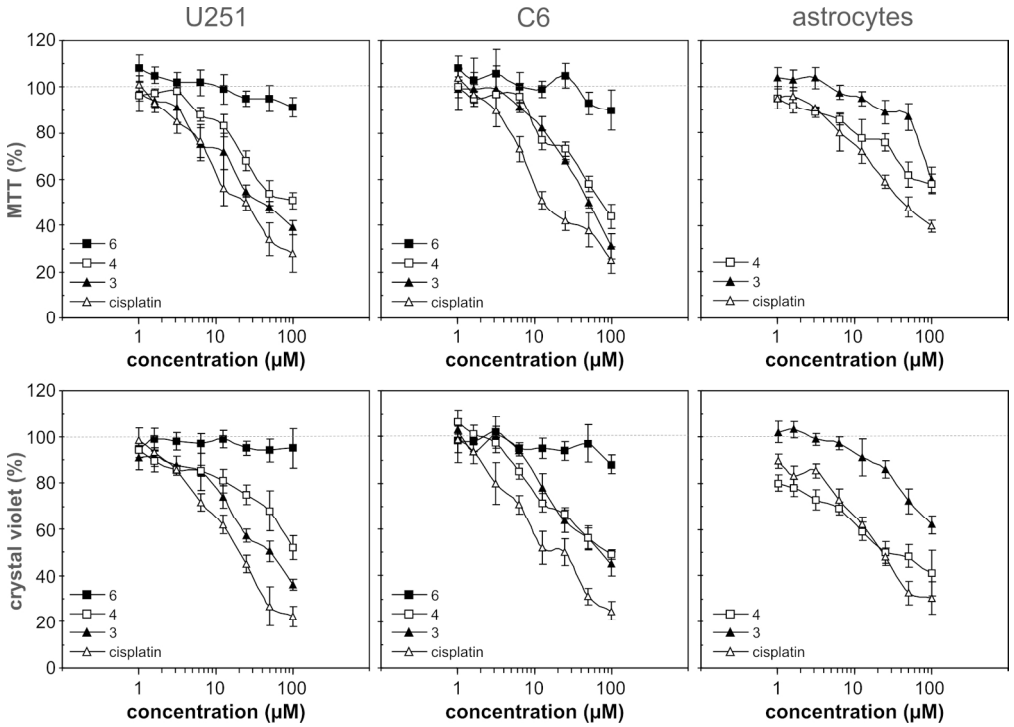


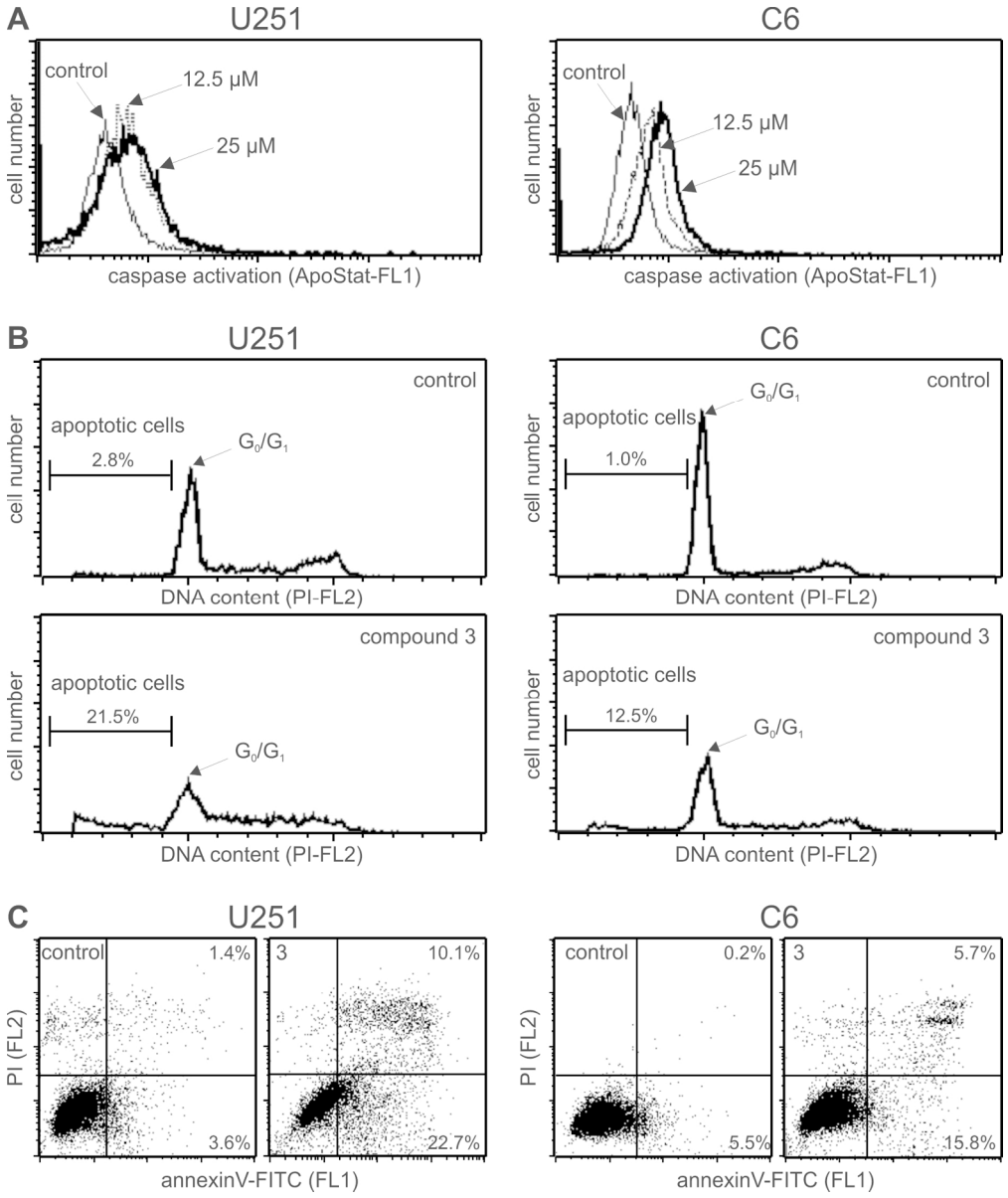
Fig. 3
64x50mm (300 x 300 DPI)

eview

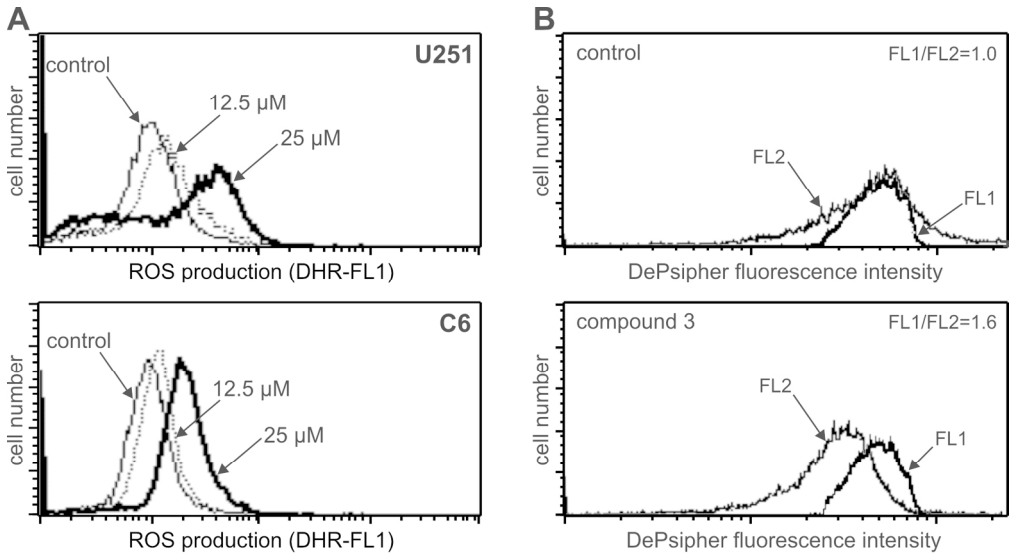


160x114mm (300 x 300 DPI)

Review



157x187mm (300 x 300 DPI)



160x87mm (300 x 300 DPI)

Peer Review