Antiproliferative and cytotoxic effects of sesquiterpene lactones isolated from Ambrosia artemisiifolia on human adenocarcinoma and normal cell lines

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ABSTRACT

Context: Ambrosia artemisiifolia (Asteraceae) contains sesquiterpene lactones as characteristic secondary metabolites. Many of these compounds exert antiproliferative and cytotoxic effects.

Objective: To isolate the sesquiterpene lactones from the aerial part of A. artemisiifolia and to elucidate their cytotoxic, antiproliferative and antibacterial effects.

Materials and methods: The compounds were identified by one-dimensional (1D) and 2D NMR, HR-MS spectroscopy from the methanol extract. Isolated compounds were investigated for their cytotoxic and antiproliferative effects on human colonic adenocarcinoma cell lines and human embryonal lung fibroblast cell line using MTT assay. The selectivity of the sesquiterpenes was calculated towards the normal cell line. To check the effect of drug interactions between compounds and doxorubicin, multidrug-resistant Colo 320 cells were used.

Results: A new seco-psilostachyinolide derivative, 1,10-dihydro-1′-noraltamisin, and seven known compounds were isolated from the methanol extract. Acetoxydihydromesanin had the most potent cytotoxic effect on sensitive (Colo205) cell line (IC50 = 7.64 μM), also the strongest antiproliferative effect on Colo205 (IC50 = 5.14 μM) and Colo320 (IC50 = 3.67 μM) cell lines. 1′-Noraltamisin (IC50 = 8.78 μM) and psilostachyin (IC50 = 5.29 μM) showed significant antiproliferative effects on the multidrug-resistant Colo320 cell line and had moderate selectivity against human embryonal lung fibroblast cell line. Psilostachyin C exhibited cytotoxic effects on Colo205 cells (IC50 = 26.60 μM). None of the isolated compounds inhibited ABCB1 efflux pump (EP; P-glycoprotein) or the bacterial EPs.

Discussion and conclusions: Acetoxydihydromasanin, 1′-noraltamisin, and psilostachyin showed the most remarkable cytotoxic and antiproliferative activity on tumour cell lines and exerted selectivity towards MRC-5 cell line.

Introduction

Common ragweed (Ambrosia artemisiifolia L. [Asteraceae]) is an annual pioneer plant; the genus stems from the Sonoran Desert (USA) according to the phylogenetic studies (Payne 1964). Common ragweed is the most widespread species of the genus and its appearance has been recorded in central (Hungary, Austria and Slovakia), eastern (Ukraine), south-eastern (Croatia and Serbia) and southern (France and Italy) parts of Europe. Although, presently, A. artemisiifolia is relatively rare in the northern part of the continent (e.g., Ireland, Scotland, Norway and Sweden), the climate change and the great genetic variability of the plant can promote the infection of these regions in the near future (Hyvönen et al. 2011). As a weed, it produces large amount of highly allergenic pollen which can induce allergic disease, such as rhinitis, conjunctivitis and asthma. The two major allergens of ragweed pollen are the Amb a I and Amb a II endopeptidases with immunoglobulin-E binding capacity (King et al. 1964; Wopfner et al. 2009). In addition, dermal exposure to the plant can cause contact dermatitis and urticaria, which has been described in other Asteraceae plants. This reaction can be attributed to the sesquiterpene lactones, representative compounds of this family (Möller et al. 2002). The widespread use of common ragweed in European folk medicine has not been documented, although ethnobotanical sources mention certain use of A. artemisiifolia by Native Americans for medicinal purposes (e.g., to treat insect bites, infected toes, minor skin eruptions, hives, tea used for fever and nausea). The medicinally used parts were the leaves and herbs. However, there are no detailed data on the developmental stages of the utilized plants (Speck 1941; Tantaquidgeon 1942; Romero 1954; Foster and Duke 1990).

Most of the Ambrosia sesquiterpenes possess of a C-11/C-13 exocyclic double bound conjugated to the γ-lactone which provides characteristic bioactivities to these secondary metabolites such as antiproliferative, cytotoxic and anti-inflammatory activities. These compounds alkylate nucleophiles, among them enzymes containing sulphhydryl groups which are crucial in cell proliferation and division and thus promote apoptosis in cancer...
cells (Kreuger et al. 2012). p65 is the specific target for sesquiterpenes, which is one of the members of the transcription factor NF-κB. This signalling pathway has a major role in tumour biology; it regulates key processes during initiation and progression of several types of cancer. In cancerous cells the NF-κB signalling promotes proliferation, angiogenesis, invasion, metastasis, chemoresistance and radio-resistance (Vazquez-Santillan et al. 2015). The tumour necrosis factor-α (TNF-α) is an activator of NF-κB. Sesquiterpene lactones damsin, ambrosin, coronopilin isolated from A. arborescens inhibited the TNF-α-induced translocation of NF-κB and all the compounds were cytotoxic to the breast cancer cell lines (MCF-7, JIMT-1 and HCC1937) (Sotillo et al. 2017). Compounds with other mechanisms of action, including G2 cell cycle checkpoint inhibitors (psilostachyin and psilostachycin C isolated from A. artemisifolia) blocked MCF-7 mp53 cells in mitosis and caused the formation of aberrant microtubule spindles (Sturgeon et al. 2005). In cancer cells, the G2 checkpoint represents the cell’s defence against DNA damage. Psilostachyin and psilostachycin C as novel G2 checkpoint inhibitors offer a potential therapeutic approach to increase the efficacy of common DNA-damaging anticancer treatments.

Our study focussed on the isolation and identification of cytotoxic and antiproliferative secondary metabolites from common ragweed (Asteraceae), the characterization of their biological activities with special attention to the selective efficacy on cancer cell lines.

Materials and methods

General experimental procedure

Analytical-grade solvents were purchased from Chem-Lab NV (Zedelgem, Belgium). Solvents for extraction were obtained from Molar Chemicals Kft. (Halásztelek, Hungary). HPLC grade solvents were acquired from VWR Chemicals International S. A. S. (Fontenay-sous-Bois, France). HPLC grade water was purified by a Millipore Direct-Q® 3 UV pump (Millipore S. A. S., Molsheim, France). Silica gel 60 RP-18 F254s and silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) were used for TLC analysis. MN-Polyamide SC-6 (polycaprolactam, 0.05–0.16 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) was applied for open column chromatography. The vacuum chromatography was carried out with a Büchi Rotavapor R-300 vacuum pump V-300 (BÜCHI Labortechnik AG, Flawil, Switzerland) using silica gel columns with a gradient system of MeOH-H2O with increasing MeOH concentration (from 20 to 100%) (17.5 L 20% MeOH, 30 L 40% MeOH, 42.5 L 60% MeOH and 55 L 80% MeOH), gaining 15 fractions (AA20/1, AA20/2, AA40/1, AA40/2, AA60/1-AA60/4, AA80/1-AA80/6 and AA100). Fraction AA20/1 (66 g) was subjected to vacuum chromatography on silica gel column with a gradient system of n-hexane-CHCl3-MeOH (50:30:0, 50:30:02, 50:30:05, 50:30:10, 50:30:20, 50:30:30, 50:30:40, 50:50:60, 50:60:70, 50:50:70, 100% MeOH) and gaining 10 fractions (AA/C1/C1-AA/C1/C10). Fraction AA/C1/C1 (3.8 g) eluted by n-hexane-CHCl3-MeOH (50:30:40) were further separated by RPC on silica gel and eluted with cyclohexane-20% MeOHAc (16:4, 14:6, 12:8, 10:10, 8:12, 100% MeOH) with a flow rate 7 mL/min gaining 7 fractions (AA/C1/C4/R1-AA/C1/C4/R7). Fraction AA/C1/C4/R2* (433 mg) was purified by RPC on silica gel (gradient elution with cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min), 4 fractions were collected (AA/C1/C4/R2*/R1-AA/C1/C4/R2*/R4). Finally, compound 5 (17 mg) was obtained from fraction AA/C1/C4/R2*/R2 (184.9 mg) by using semipreparative HPLC gradient solvent system, consisting of methanol and water (0 min: MeOH-H2O 6:5:3.5, 6 min: MeOH:H2O 6:5:3.5, 9 min:MeOH:H2O 8:2, 10 min:MeOH:H2O 1:0, 11 min: MeOH:H2O 6:5:3.5, flow rate 2.8 mL/min)). Fraction AA/C1/C4/R6* (400 mg) was subjected to RPC using silica gel with gradient elution (cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min). Two fractions were collected (AA/C1/C4/R6*/R1-182.4 mg) by using semipreparative HPLC gradient solvent system, consisting of methanol and water (0 min: MeOH-H2O 6:5:3.5, 6 min: MeOH:H2O 6:5:3.5, 9 min:MeOH:H2O 8:2, 10 min:MeOH:H2O 1:0, 11 min: MeOH:H2O 6:5:3.5, flow rate 2.8 mL/min)). Fraction AA/C1/C4/R6*/R1 (213.3 mg) and AA/C1/C4/R6*/R2 (138.4 mg). Fraction AA/C1/C4/R6*/R1 (213.3 mg) was dissolved in MeOH-dichloromethane 9:1, stored in the refrigerator for 24 h. Compound 2 (35 mg) was obtained by crystallization from this solvent mixture. Fraction AA/C1/C4/R5* (385 mg) was separated by RPC on silica gel with the same method gaining two fractions, AA/C1/C4/R5*/R1 (177.5 mg) and AA/C1/C4/R5*/R2 (161.1 mg). Fraction AA/C1/C4/R5*/R2 (161.1 mg) was further separated by RPC on silica gel eluting with tolune-acetone (18:2, 17:3, 16:4, 15:5, 100% MeOH in the final step), affording 6 fractions (AA/C1/C4/R5*/R2/R1-AA/C1/C4/R5*/R2/R6).
Fraction AA/C1/C4/R5*/R2/R1 (10.5 mg) was purified by semi-preparative HPLC (gradient elution, MeOH-H₂O system, 0–1 min: MeOH-H₂O 40:60, 1–12 min: MeOH-H₂O 40 → 100, 12–13 min: MeOH-H₂O 100:0, 13–14 min: MeOH-H₂O 100 → 40, flow rate 3.0 mL/min) to obtain compound 3 (6 mg). Fraction AA/C1/C4/R4* (380 mg) was subjected on silica gel and separated by RPC (gradient elution, cyclohexane-dichloromethane-MeOH 16:4:0.5, 14:5:0.5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min). Three fractions were collected (AA/C1/C4/R4*/R1-AA/C1/C4/R4*/R2). Fraction AA/C1/C4/R4*/R1 (104 mg) was dissolved in MeOH-dichloromethane 9:1, stored in the refrigerator for 24 h. Compound 6 (27 mg) was obtained by crystallization form this solvent system. Fraction AA/C1/C4/R4*/R2 (71.2 mg) was subjected to semipreparative HPLC (gradient solvent system, 0–1 min: MeOH-H₂O 40:60, 1–10 min: MeOH 40 → 60, 10–12 min: MeOH-H₂O 60:40, 12–15 min: MeOH 60 → 100, 15–16 min MeOH-H₂O 100:0, 16–17 min: MeOH 100 → 40, flow rate 2.8 mL/min) to obtain compound 4 (34 mg). Fraction AA/C1/C4/R3* (415.2 mg) was purified by RPC using silica gel (gradient elution, cyclohexane-dichloromethane-MeOH 16:4:0:5, 14:5:0:5, 14:5:1, 12:7:1, 100% MeOH in the final step) and gaining 4 fractions (AA/C1/C4/R3*/R1-AA/C1/C4/R3*/R4). Fraction AA/C1/C4/R3*/R2 (149.9 mg) was further separated by RPC on silica gel (gradient elution, toluene-acetone 18:2, 17:3, 16:4, 15:5, 100% MeOH in the final step, flow rate 7 mL/min). Four fractions were collected (AA/C1/C4/R3*/R2/R1-AA/C1/C4/R3*/R2/ R4). Subfraction AA/C1/C4/R3*/R2/R2 (30 mg) was further separated by semipreparative HPLC (gradient solvent system, 0–1 min: MeOH-H₂O 40:60, 1–10 min MeOH 40 → 60, 10–12 min: MeOH-H₂O 64:40, 12–15 min: MeOH 60 → 100, 15–16 min: MeOH-H₂O 100:0, 16–17 min: MeOH 100 → 40, flow rate 2.8 mL/min) to obtain compound 7 (4 mg). Fraction AA/C1/C6 (4.12 g) was subjected to RPC with gradient elution system (cyclohexane-dichloromethane-MeOH 16:4:0:5, 14:5:0:5, 14:5:1, 12:7:1, 100% MeOH in the final step, flow rate 7 mL/min). Six fractions were collected (AA/C1/C6/R2/R1-AA/C1/C6/R2/R3/R4). Fraction AA/C1/C6/R1 (126 mg) was further separated by RPC in the same way to gain five fractions (AA/C1/C6/R3*/R1-AA/C1/C6/R3*/R3/R5). Subfraction AA/C1/C6/R3*/R1 (69 mg) was purified by HPLC (Shimadzu LC-10AS system, isocratic elution, MeOH-H₂O 60:40, flow rate 0.8 mL/min) to obtain 1 (2 mg). Fraction AA/60/3 (3.4229 g) was chromatographed by open column chromatography on silica gel with a gradient system of n-hexane-EtOAc (9:1, 8:2, 4:1, 1:1, 1:4, 1:9 and 0:1) and EtOAc-MeOH (9:1, 4:1, 1:1, 1:4, 1:9 and 0:1) gaining 6 fractions (AA60/3/C1-AA60/3/C6). Subfraction AA60/3/C5 (322 mg) was further separated by size-exclusion chromatography (Sephadex LH-20) with MeOH gaining seven subfractions (AA60/3/C5/S1-AA60/3/C5/S7). Compound 8 (34 mg) was obtained in subfraction AA60/3/C5/S7.

**Cell lines**

Human colon adenocarcinoma cell lines (Colo 205 doxorubicin-sensitive and Colo 320/MDR-LRP multidrug-resistant expressing ABCB1 (MDR1)-LRP), ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205) were purchased from LGC Promochem, Teddington, UK. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM l-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cell lines were incubated at 37°C, in a 5% CO₂, 95% air atmosphere. The semi-adherent human colon cancer cells were detached with Trypsin-Versene (EDTA) solution for 5 min at 37°C.

MRC-5 human embryonal lung fibroblast cell lines (ATCC CCL-171) were purchased from LGC Promochem, Teddington, UK. The cell line was cultured in Eagle's Minimal Essential Medium (EMEM, containing 4.5 g/L glucose) supplemented with a non-essential amino acid mixture, a selection of vitamins and 10% heat-inactivated foetal bovine serum. The cell lines were incubated at 37°C, in a 5% CO₂, 95% air atmosphere.

**Bacterial strains and determination of MIC values**

As Gram-positive strains, *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 strain was used as methicillin-susceptible reference strain; the clinical isolate *S. aureus* MRSA 272123 and *Enterococcus faecalis* ATCC 29212 strains were investigated in the study.

As Gram-negative strains *Escherichia coli* ATCC 25922 and *E. coli* AG100; *Klebsiella pneumoniae* ATCC 49619 and *Pseudomonas aeruginosa* ATCC 33362 strains were used. The MICs of compounds were determined, according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute 2022).

**Assay for antiproliferative and cytotoxic effects**

MRC-5 non-cancerous human embryonic lung fibroblast and human colon adenocarcinoma cell lines (doxorubicin-sensitive Colo 205 and multidrug-resistant Colo 320 colon adenocarcinoma cells) were used to determine the effect of compounds on cell proliferation and growth. The effects of increasing concentrations of compounds on cell proliferation and growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 100 µL of medium.

The adherent human embryonal lung fibroblast cells were seeded overnight and cultured in 96-well flat-bottomed microtitre plates prior to the assay, using EMEM supplemented with 10% heat-inactivated foetal bovine serum. The density of the cells was adjusted to 6 × 10⁴ cells (cytotoxicity assay) or 24 h (cytotoxicity test); at the end of the incubation period, 20 µL of MTT (thiazolyl blue tetrazolium bromide, Sigma, St. Louis, MO, USA) solution (from a stock solution of 5 mg/mL) were added to each well. After incubation at 37°C for 4 h, 100 µL of sodium dodecyl sulphate (SDS) (Sigma) solution (10% in 0.01 M HCl) were added to each well, with the exception of the medium control wells. The final volume of the wells containing compounds and cells was 200 µL.

The culture plates were incubated at 37°C for 72 h (antiproliferative test) or 24 h (cytotoxicity test); at the end of the incubation period, 20 µL of MTT (thiazolyl blue tetrazolium bromide, Sigma, St. Louis, MO, USA) solution (from a stock solution of 5 mg/mL) were added to each well. After incubation at 37°C for 4 h, 100 µL of sodium dodecyl sulphate (SDS) (Sigma) solution (10% in 0.01 M HCl) were added to each well and the plates were further incubated at 37°C overnight. Cell growth was determined by measuring the optical density (OD) at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems,
Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula below:

\[
\text{Inhibition} \% = 100 - \frac{\text{OD sample} - \text{OD medium control}}{\text{OD cell control} - \text{OD medium control}} \times 100
\]

Results are expressed in terms of IC\text{50} defined as the inhibitory dose that reduces the growth of the cells exposed to the tested compounds by 50%.

**Evaluation of rhodamine 123 (R123) retention by flow cytometry (inhibition of P-glycoprotein or ABCB1)**

This method is a fluorescence-based detection system which uses tariquidar as a reference inhibitor of the ABCB1 efflux pump (EP). The colonic adenocarcinoma cells (the doxorubicin-sensitive Colo 205 and the multidrug-resistant Colo 320) were adjusted to a density of \(2 \times 10^6\)/mL, re-suspended in serum-free RPMI 1640 medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The compounds were added at different concentrations (2 \(\mu\)M and 20 \(\mu\)M final concentrations, respectively), and the samples were incubated for 10 min at room temperature. Next, 10 \(\mu\)L (5.2 \(\mu\)M final concentration) of rhodamine 123 was added to the samples and the cells were incubated for 20 minutes at 37°C, washed twice with phosphate-buffered saline (PBS) and re-suspended in 1 mL PBS for analysis. The fluorescence intensity of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Munster, Germany). Tariquidar was used as a positive control at 0.2 \(\mu\)M final concentration in the rhodamine 123 exclusion experiments. The mean fluorescence intensity (%) was calculated for the treated MDR Colo 320 and sensitive Colo 205 cell lines as compared to the untreated cells. The fluorescence activity ratio (FAR) was calculated based on the following equation which relates the measured fluorescence values:

\[
\text{FAR} = \frac{\text{Colo 320 treated}}{\text{Colo 320 control}} \times \frac{\text{Colo 205 control}}{\text{Colo 205 treated}}
\]

**Checkerboard combination assay**

A checkerboard microplate method was applied to study the effect of drug interactions between the compounds and doxorubicin. The assay was carried out using multidrug-resistant Colo 320 colonic adenocarcinoma cells expressing the ABCB1 transporter. The final concentration of the chemotherapeutic agent doxorubicin used in the combination experiment was chosen in accordance with their antiproliferative activity previously determined. The dilutions of the chemotherapeutic drug were made in a horizontal direction in 100 \(\mu\)L aliquots into Eppendorf centrifuge tubes. The compounds were added at different concentrations (2 \(\mu\)M and 20 \(\mu\)M final concentrations, respectively), and the samples were incubated for 10 min at room temperature. Next, 10 \(\mu\)L (5.2 \(\mu\)M final concentration) of rhodamine 123 was added to the samples and the cells were incubated for 20 minutes at 37°C, washed twice with phosphate-buffered saline (PBS) and re-suspended in 1 mL PBS for analysis. The fluorescence intensity of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Munster, Germany). Tariquidar was used as a positive control at 0.2 \(\mu\)M final concentration in the rhodamine 123 exclusion experiments. The mean fluorescence intensity (%) was calculated for the treated MDR Colo 320 and sensitive Colo 205 cell lines as compared to the untreated cells. The fluorescence activity ratio (FAR) was calculated based on the following equation which relates the measured fluorescence values:

\[
\text{FAR} = \frac{\text{Colo 320 treated}}{\text{Colo 320 control}} \times \frac{\text{Colo 205 control}}{\text{Colo 205 treated}}
\]

**Real-time ethidium bromide accumulation assay**

The minimum inhibitory concentrations (MICs) of the substances were determined as a prelude to the experiment. The substances did not have a significant effect on any of the bacterial strains. The MIC value was \(>100 \mu\)M in all cases. The bacteria were incubated in Mueller–Hinton (MH) broth.

The impact of compounds on EB accumulation was determined by the automated EB method using a CLARIOstar Plus plate reader (BMG Labtech, Aylesbury, UK). First, the bacterial strain was incubated until it reached an OD of 0.6 at 600 nm [Escherichia coli AG100 is incubated in Luria–Bertani (LB) broth and Staphylococcus aureus ATCC 25923 is incubated in tryptic soy broth (TSB)]. The culture was washed with PBS (pH 7.4) and centrifuged at 13,000 \(\times\) g for 3 min, the cell pellet was re-suspended in PBS. The compounds were added at 1/2 MIC concentration (in case of \(>100\) the concentration is 100 \(\mu\)M) to PBS containing a non-toxic concentration of EB (2 \(\mu\)g/mL). Then, 50 \(\mu\)L of the EB solution containing the compound were transferred into 96-well black microtitre plate (Greiner Bio-One Hungary Kft, Hungary), and 50 \(\mu\)L of bacterial suspension (OD\text{600} 0.6) were added to the each well. Then, the plates were placed into the CLARIOstar plate reader, and the fluorescence was monitored at excitation and emission wavelengths of 525 and 615 nm every minute for 1 h on a real-time basis. From the real-time data, the activity of the compounds, namely the relative fluorescence index (RFI) of the last time point (60 min) of the EB accumulation assay, was calculated according to the following formula:

\[
\text{RFI} = \frac{(\text{RF treated} - \text{RF untreated})}{\text{RF untreated}}
\]

where RF treated is the relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor, and RF untreated is the RF at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). (We also had a negative control: bacteria and EB solution only.)

Positive controls: E. coli AG100 \(\rightarrow\) CCCP (50 \(\mu\)M) S. aureus ATCC 25923 \(\rightarrow\) reserpine (25 \(\mu\)M)

**Results**

**Structure determination of the isolated compounds**

The structure elucidation process was carried out by 1D (\(^1\)H, \(^{13}\)C JMOD) and 2D (HSQC, HMBC, \(^{1}H-^{1}H\) COSY and NOESY) NMR experiments. By comparison of the spectroscopic data with the literature data, the seven known isolated compounds were identified as psilostachyin C (1) (Kagan et al. 1966), acetoxydihydrodamsin (2) (Tagliatela-Scafati et al. 2012), peruvin (3) (Oberti et al. 1986), psilostachyin (4) (Borges-del-Castillo et al.
Compound 7 was isolated as a white amorphous powder. The molecular formula was identified as C_{16}H_{24}O_{5} by HR-ESIMS at m/z 297.16947 [M + H]^+ (calcd for C_{16}H_{25}O_{5} 297.16965), indicating 5 unsaturated degrees. The 1H-NMR spectrum of 7 revealed two methyl groups, one methoxy, one unsaturated methylene, and one oxygenated methine (Table 1). The 13C-JMOD spectrum, together with the assistance of HSQC and HMBC spectra of 7, indicated 16 carbon signals, including three methyls, one olefinic methylene, four sp^3 methylenes, four sp^3 methines, one olefinic quaternary carbon, one oxygenated quaternary carbon and two carbonyl carbons (Table 1). The 1H-1H COSY together with HSQC spectra of 7 clearly revealed the COSY correlations of CH-1 (δ_{H} 1.59, m 53.0)/CH_{2}-2 (δ_{H} 1.46; δ_{C} 21.8)/CH_{2}-3 (δ_{H} 2.35, 2H; δ_{C} 36.4)/CH-6 (δ_{H} 4.55; δ_{C} 89.6)/CH-7 (δ_{H} 3.49, m 41.9)/CH_{2}-8 (δ_{H} 2.18; δ_{C} 36.3)/CH_{2}-9 (δ_{H} 5.59; δ_{C} 141.0) (Figure 2). Furthermore, the HMBC correlations from H_{2}-2, H_{2}-3, and a methoxy group (δ_{H} 3.65, 3H; δ_{C} 52.0) to the carbonyl carbon C-4 (δ_{C} 175.9), respectively, indicated a methyl propionate moiety located at C-1. In addition, the HMBC correlations from H-6 to the olefinic quaternary carbon C-11 (δ_{H} 141.0) and another carbonyl carbon C-12 (δ_{C} 172.6), and from H_{2}-13 to C-7, C-11 and C-12, indicated an α-methylene-γ-butyrolactone system connected at C-6 and C-7 in existence (Figure 2). Based on the above analysis, compound 7 can be identified as a rare seco-psilostachyinolide (Borges-del-Castillo et al. 1981; Delgado et al. 1988) and similar structures (Oberti et al. 1986; Delgado et al. 1988) suggested that the planar structure of 7 was the same as the psilostachyin C derivative. However, the psilostachyin C derivative was only reported as a synthetic product without any structural elucidation and was never named in the literature (Kagan et al. 1966). In this work, we successfully obtained compound 7 from natural sources and completely revealed its NMR spectra data and structural analysis for the first time. Further, the NOESY cross-peaks between H-1/H-6/H-7/H-10 and H-2 (δ_{H} 1.92)/H_{3}-15/H_{3}-14, as well as comparing with psilostachyin C (Kagan et al. 1966), indicated the relative configuration of 7 as shown on Figure 1 and named as 1,10-dihydro-1'-noraltamisin.

**Antibacterial effects of compounds**

None of the tested compounds exerted antibacterial effect. The MIC values on Gram-negative and -positive bacteria were above 100 μM.
Antiproliferative and cytotoxic assays

To assess the in vitro antiproliferative and cytotoxic activity of the isolated compounds a MTT assay was performed using two human adenocarcinoma cell lines (the doxorubicin-sensitive Colo 205 and the multidrug-resistant Colo 320 colonic adenocarcinoma cell lines). Compound 2 exhibited cytotoxic activity (IC_{50} value of 7.64 ± 0.37 μM) on sensitive (Colo 205) cell line after 24 h of incubation (Table 2). Among the seco-psilostachyinolides, 1 showed the strongest cytotoxic effects with IC_{50} values of 26.6 ± 0.48 μM on the doxorubicin-sensitive Colo 205 cell line, while compound 4 had cytotoxic effects on the multidrug-resistant Colo 320 cell line (IC_{50} value 17.7 ± 0.20 μM). In contrast, compound 6 which shares the same seco derivative skeleton had no cytotoxic effects with the tested cell lines (Table 2). Besides the isolated sesquiterpenes the quercetagetin 3,6-dimethyl ether derivative (8) showed no cytotoxic effects on the tested cell lines after a short time exposure (Table 2).

After a 72 h incubation period, compound 2 had the strongest antiproliferative activity among the isolated sesquiterpenes on both adenocarcinoma cell lines (IC_{50} value of 5.14 ± 0.55 μM on Colo 205 and IC_{50} value of 3.67 ± 0.35 μM on Colo 320, Table 3). Compound 6 was inactive in this assay as well, however, compound 8 inhibited the cell proliferation of the human embryonal lung fibroblast cells at very low concentration (IC_{50} value of 4.03 ± 0.56 μM) (Table 3).

Compound 2 exerted selective cytotoxicity towards the human colonic adenocarcinoma cell lines in comparison with its activity against human embryonal lung fibroblast cell line (selectivity index [SI] = 3.11). In contrast, compounds 4 and 6 showed cell proliferation inhibitory activities on the Colo320 multidrug-resistant cell line with IC_{50} values of 8.78 ± 0.22 μM, 5.29 ± 0.15 μM, and the molecules had relatively moderate selectivity against the MRC-5 cell line (SI = 3.04 and SI = 4.98).

Checkboard combination assay

The drug interactions between the compounds and doxorubicin were assessed using multidrug-resistant Colo320 colonic adenocarcinoma cells which expressing the ABCB1 transporter. Compounds 7 and 8 have synergistic effects with the doxorubicin at certain concentrations. However, compounds 1–3 showed antagonistic effects at certain concentrations (Table 4).

ABC21 efflux pump (P-glycoprotein) inhibitory assay

In this study, we used tariquidar (FAR value: 6.6 at 0.2 μM) as a reference inhibitor of the P-glycoprotein EP expressed by the two adenocarcinoma cell lines. The fluorescence dye, rhodamine 123 was used as a marker of the ABCB1 transporter activity to explore the potential inhibitory activity of the isolated compounds. Inside cells without overexpressing the P-glycoprotein, the rhodamine 123 accumulates and results in an increase in fluorescence intensity (F_{em}/F_{ex} = 490/525 nm). The multidrug resistance (MDR) cells overexpressing the EP can easily remove the dye which results the rapid decreasing the intensity of cellular fluorescence. If the fluorescence activity ratio (FAR) value is above 2, it is conventionally accepted that the subjected compound can be a good ABCB1 inhibitor. After measuring the fluorescence intensity of the cell population none of the compounds showed adequate inhibitory effects according to their FAR values. The FAR values of the tested compounds were in the range of 0.2-1.1.

The ethidium bromide (EB) accumulation assay

The EB accumulation assay gives insight information about the function of the EP expressed by Escherichia coli AG100 and Staphylococcus aureus ATCC 25923. The effective EP inhibitors increase the level of fluorescence of the intercalating agent EB (used as fluorescence dye) because of its accumulation within the bacteria. The EP inhibiting activity of the isolated compounds was evaluated based on the RFI of the real-time accumulation curves. In this study, none of the sesquiterpenes showed EP inhibitory activity. The RFI values were around 0, that is, equal to the untreated sample. RFI values obtained for positive controls are as follows: reserpine (RFI = 2.77) on S. aureus ATCC 25923 and CCCP (carbonyl cyanide m-chlorophenylhydrazine) (RFI = 1.34) on E. coli AG100.

Discussion

Eight compounds were isolated from the MeOH extract of the aerial parts of A. artemisiifolia and based on HR-MS and NMR...
experiments their structures were identified as the sesquiterpenes psilostachyin C (1), acetoxoydrodamsin (2), peruvin (3), psilostachyin (4), 1’-noraltamin (5), psilostachyin B (6), 1,10-dihydro-1’-noraltamin (7) and the flavonoid axillarin (8). After comparing NMR data of 1,10-dihydro-1’-noraltamin (7) with those found in the literature, it was realized that this compound was obtained for the first time from the plant. It belongs to a rare class of seco derivatives bearing an open ring system. It can be hypothesized that the biosynthesis of this class of seco derivatives takes place in two independent pathways: the mevalonic acid (MVA) pathway in the cytoplasm or the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway in the plastids (Bick and Lange 2003). The precursor in both cases, 2x,6x-farnesyl pyrophosphate (FPP), results from the addition of a molecule of IPP onto GPP. The skelota of this open ring system arise from the cyclodecadiene-type product of the cyclization of FPP, or of its geometrical isomer at C-2 (2z,6x-FPP), or of norerolidyl pyrophosphate, by nucleophilic attack on the distal double bond which leads to the germacradienyl cation, from which may arise also the other lactone skeletal types (Jean 1999). Until now just a few of this type of seco derivatives were reported in the literature from the genus Ambrosia. 1’-Noraltamin (5) was reported only from a Mexican population (collected in North Zacatecas) of Ambrosia confertiflora DC (Delgado et al. 1988). Structurally similar sesquiterpene lactones, altamisin from A. peruviana (Central American collection) (Borges et al. 1978) and altamisin acid from A. tenuifolia (collection form North Central Argentina), were also reported (Oberti et al. 1986). From a Yugoslavian collection of A. artemisiifolia, Stefanovic et al (1987) isolated 4-oxo-3,4-seco-ambrosan-6,12-olide-3-oic acid; the same compound was also found by Taglialatela-Scafati et al. (2012) from an Italian plant sample.

The cytotoxic and antiproliferative activities of the sesquiterpene lactones are the most characteristic bioactivities in this naturally occurring secondary metabolites. To assess these activities, we used two different experimental setups of MTT assay. This allows the comparison of results obtained from a short-term (24 h) treatment of a relatively higher number of cells (cytotoxic activity) and those from a long-term (72 h) treatment of a lower cell number (cytostatic or antiproliferative activity). In the

### Table 4.
The drug interactions between the compounds and doxorubicin.

| Compounds Starting concentration | Ratio* | CI at ED<sub>50</sub> | SD | Type of interaction |
|---------------------------------|--------|---------------------|----|-------------------|
| 1 50 μM                          | 5.8:1  | 3.71                | 0.73| Strong antagonism  |
|                                 | 11.6:1 | 3.34                | 0.43| Strong antagonism  |
|                                 | 23.2:1 | 2.55                | 0.31| Antagonism         |
|                                 | 46.4:1 | 1.13                | 0.07| Slight antagonism  |
|                                 | 92.8:1 | 1.47                | 0.28| Antagonism         |
|                                 | 185:1  | 1.34                | 0.28| Moderate antagonism|
| 2 15 μM                          | 1.7:4:1| 2.45                | 0.48| Antagonism         |
|                                 | 3.48:1 | 2.37                | 0.16| Antagonism         |
|                                 | 6.96:1 | 1.48                | 0.22| Antagonism         |
|                                 | 13.92:1| 1.55                | 0.15| Antagonism         |
|                                 | 27.84:1| 1.22                | 0.23| Moderate antagonism|
|                                 | 55.68:1| 1.34                | 0.29| Moderate antagonism|
| 3 65 μM                          | 7.5:4:1| 1.28                | 0.16| Moderate antagonism|
|                                 | 15.08:1| 1.7                 | 0.23| Antagonism         |
|                                 | 30.16:1| 2.34                | 0.1  | Antagonism         |
|                                 | 60.32:1| 1.4                 | 0.024| Moderate antagonism|
|                                 | 120:64:1| 1.7                | 0.11| Antagonism         |
|                                 | 241:28:1| 1.01               | 0.18| Additive effect    |
| 4 25 μM                          | 2.9:1  | 0.95                | 0.06| Additive effect    |
|                                 | 5.8:1  | 1.44                | 0.32| Moderate antagonism|
|                                 | 11.6:1 | 0.67                | 0.14| Synergism          |
|                                 | 23.2:1 | 2.26                | 0.42| Antagonism         |
|                                 | 46.4:1 | 1.39                | 0.07| Slight antagonism  |
|                                 | 92.2:1 | 1.16                | 0.21| Slight antagonism  |
| 5 30 μM                          | 3.48:1 | 2.62                | 0.62| Antagonism         |
|                                 | 6.69:1 | 1.02                | 0.072| Additive effect   |
|                                 | 13.92:1| 0.88                | 0.043| Slight synergism  |
|                                 | 27.84:1| 1.09                | 0.039| Additive effect   |
|                                 | 55.68:1| 1.33                | 0.036| Moderate antagonism|
|                                 | 111.36:1| 0.94               | 0.28| Additive effect    |
| 6 100 μM                         | 11.6:1 | 0.4                 | 0.14| Syn.               |
|                                 | 23.2:1 | 0.34                | 0.06| Syn.               |
|                                 | 46.4:1 | 2.6                 | 0.36| Antagonism         |
|                                 | 92.8:1 | 1.13                | 0.09| Slight antagonism  |
|                                 | 185.6:1| 1.4                 | 0.2  | Moderate ant.      |
|                                 | 371.2:1| 1.5                 | 0.16| Antagonism         |
| 7 150 μM                         | 17.4:1 | 0.1                 | 0.03| Very strong syn.   |
|                                 | 34.84:1| 0.79                | 0.02| Moderate syn.      |
|                                 | 69.68:1| 0.74                | 0.03| Moderate syn.      |
|                                 | 139.36:1| 0.74               | 0.05| Moderate syn.      |
|                                 | 278.72:1| 0.75               | 0.7  | Moderate syn.      |
|                                 | 557.44:1| 0.51               | 0.05| Syn.               |

*Ratio: the best combination ratio between compounds and doxorubicin.
CI at ED<sub>50</sub>: combination index value at the 50% growth inhibition dose.

Combination index (CI): 0–0.1: very strong synergism, 0.1–0.3: strong synergism, 0.3–0.7: synergism, 0.7–0.85: moderate synergism, 0.85–0.9: slight synergism, 0.9–1.1: additive effect, 1.1–1.2: slight antagonism, 1.2–1.45: moderate antagonism, 1.45–3.3: antagonism, 3.3–10: strong antagonism and >10: very strong antagonism.
in vitro studies, acetoxydihydrodamsin (2) showed the most potent cytotoxic effect on sensitive (Colo 205) cell line. After a long-term exposure to the above-mentioned compound, a strong antiproliferative effect was also observed on Colo205 and Colo320 adenocarcinoma cell lines. The seco-psilostachyinolides, 1’-noraltamisin (5) and psilostachyin (4) have significant antiproliferative effects on the multidrug-resistant Colo 320 cell line and showed moderate selectivity against human embryonal lung fibroblast cell line. It is generally accepted that the presence of a C-11/C-13 exocyclic double bound conjugated to the γ-lactone is crucial for cytotoxicity of this type of compounds and as noted by several authors due to this moiety these secondary metabolites can alkylate the nucleophilic groups of enzymes (Kreuger et al. 2012). In spite all of these, in the case of acetoxydihydrodamsin (2), the lack of this moiety is conspicuous, compared to the other sesquiterpenes. Instead of this, compound 2 bears an acetyl group at C-3, which demonstrates that the presence of an α,β-unsaturated carbonyl group is not required for the inhibition of cell proliferation. In addition to the isolated sesquiterpene lactones, the quercetagetin 3,6-dimethyl ether derivative axillarin (8) showed antiproliferative effects on MRC-5 cells with surprisingly low concentration and no selectivity towards to this normal cell line, compared to the sesquiterpenes. Cancer cells can develop MDR against to a broad spectrum of chemotherapeutic agents which results failure in the treatment of cancer and a major challenge in the drug development to overcome this problem. The MDR cells overexpressing the ATP binding ABC transmembrane proteins can easily efflux various chemically diverse or potentially dangerous compounds across the cell membrane, e.g., anticancer drugs, which leads to chemoresistance (Gottesman and Ambudkar 2001; Dean and Annilo 2005). The ABCB1 EP the member of this transmembrane proteins can reduce the cellular uptake of drugs into cancer cells and protect them from medical interventions (Doyle et al. 1998). Naturally occurring substances which may inhibit this type of ABC transporters have the potential to amplify the efficacy of anticancer drug. In the P-glycoprotein (ABCB1) inhibitory assay none of the tested sesquiterpenes, nor the flavonoid derivative have considerable effects on the ABCB1 EP function. These findings suggest that this type of plant secondary metabolite expresses their inhibitory activity inside the cancer cells via other mechanisms, e.g., inhibition of DNA methylation (Liu et al. 2009), inhibition of the NF-kB signalling pathway (Kreuger et al. 2012), cell cycle checkpoint inhibition (Sturgeon et al. 2005), inducing apoptosis and cell cycle arrest (Martino et al. 2015).

After the EB accumulation assay, we concluded that the isolated Ambrosia sesquiterpenes could not be considered as effective bacterial EP inhibitors.

Even though the investigated Asteraceae species is an invasive and highly allergenic weed, the plant’s secondary metabolites dispose noteworthy cytotoxic and antiproliferative activity on the investigated adenocarcinoma cell lines. However, further study is still necessary to elucidate the structure–activity relationships and the molecular mechanisms of these compounds. Also, chemical modifications and semisynthetic derivatives may lead more effective and selective substances towards cancer cells.

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No potential conflict of interest was reported by the author(s).

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References
Ahmad VU, Rasool N, Abbasi MA, Rashid MA, Kousar F, Zubair M, Ejaz A, Choudhary MI, Tareen RB. 2006. Antioxidant flavonoids from Pulicaria undulata. Chem Inform. 37(754–751).

Bick JA, Lange BM. 2003. Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. Arch Biochem Biophys. 415(2):146–154.

Borges J, Manresa MT, Martín JL, Pascual C, Vázquez P. 1978. Altamisin, a new sesquiterpene lactone from H.B.K. Tetrahedron Lett. 19(17): 1513–1514.

Borges-del-Castillo J, Manresa-Ferrero MT, Rodríguez-Luis F, Vázquez-Bueno P, Joseph-Nathan P. 1981. 13C NMR study of psilostachyinolides from some Ambrosia species. Org Magn Reson. 17(3):232–234.

Clinical & Laboratory Standards Institute. 2022. [Internet] Clinical & laboratory standards institute [accessed 2022 Jan 12]. https://clsi.org/.

Dean M, Annilo T. 2005. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annu Rev Genomics Hum Genet. 6:123–142.

Delgado G, Chavez MI, Alvarez L. 1988. New seco-psuedogauaianolides and other terpenoids from a population of Ambrosia confertiflora. J Nat Prod. 51(1):83–87.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA. 95(26):15665–15670.

Foster S, Duke JA. 1990. A field guide to medicinal plants. Eastern and Central North America, Boston (MA): Houghton Mifflin Company.

Gottesman MM, Ambudkar SV. 2001. Overview: ABC transporters and human disease. J Bioenerg Biomembr. 33(6):453–458.

Hyövönen T, Glemnitz M, Radics I, Hoffmann J. 2011. Impact of climate and land use type on the distribution of Finnish causal arable weeds in Europe: distribution of Finnish causal weeds. Weed Res. 51(2):201–208.

Jean B. 1999. Pharmacognosy phytochemistry medicinal plants. 2nd ed. Paris, France: Lavoisier Publishing.

Kagan HB, Miller HE, Renold W, Lakshmanikanth MV, Tether LR, Herrz W, Mabry T. 1966. The structure of psilostachyin C, a new sesquiterpene dilactone from Ambrosia psilostachya DC. J Org Chem. 31(5):1629–1632.

King TP, Norman PS, Connell JT. 1964. Isolation and characterization of allergens from ragweed pollen. J. Biochemistry. 3:458–468.

Liu Z, Liu S, Xie Z, Pavlovicze RE, Wu J, Chen P, Anniuwwu J, Pang J, Bhasin D, Neviani P, et al. 2009. Modulation of DNA methylation by a sesquiterpene lactone parthenolide. J Pharmacol Exp Ther. 329(2):505–514.

Martino R, Beer MF, Elso O, Donadel O, Silsen V, Anesinis C. 2015. Sesquiterpene lactones from Ambrosia spp. are active against a murine lymphoma cell line by inducing apoptosis and cell cycle arrest. Toxicol In Vitro. 29(7):1529–1536.

Möller H, Spír a, Svensson Å, Gruverberger B, Hindsén M, Bruze M. 2002. Contact allergy to the Asteraceae plant Ambrosia artemisifolia L. (ragweed) in Ambrosia-parthenolide-sensitive patients in southern Sweden: ragweed contact allergy. Contact Dermatitis. 47(3):157–160.

Oberti JC, Silva GL, Sosa VE, Kulanthaveli P, Herz W. 1986. Ambrosanolides and secacombsanolides from Ambrosia tenuifolia. Phytochemistry. 25(6):1355–1358.

Kreuger MRO, Grootjans S, Biavatti MW, Vandenabeele P, D’Herde K. 2012. Sesquiterpene lactones as drugs with multiple targets in cancer treatment: focus on parthenolide. Anticancer Drugs. 23(9):883–896.

Payne WW. 1964. A re-evaluation of the genus Ambrosia (Compositae). J Arnold Arbor. 45(4):401–438.

Romero JB. 1954. The botanical lore of the California Indians. New York (NY): Vantage Press Inc.

Sotillo WS, Villagomez R, Smiljanic S, Huang X, Malakpour A, Kempengren S, Rodrigo G, Almanza G, Sterner O, Oredsson S. 2017. Anti-cancer stem cell activity of a sesquiterpene lactone isolated from Ambrosia arborescens and of a synthetic derivative. PLoS One. 12(9):e0184304–16.

Speck FG. 1941. A list of plant curatives obtained from the Houma Indians of Louisiana. Primit Man. 14(4):49–73.
Stefanović M, Aljančić-Solaja I, Milosavljević S. 1987. A 34-seco-ambrosanolide from Ambrosia artemisiifolia. Phytochemistry. 26(3):850–852.

Sturgeon CM, Craig K, Brown C, Rundle NT, Andersen RJ, Roberge M. 2005. Modulation of the G2 cell cycle checkpoint by sesquiterpene lactones psilostachyins A and C isolated from the common ragweed Ambrosia artemisiifolia. Planta Med. 71(10):938–943.

Tagliatela-Scafati O, Pollastro F, Minassi A, Chianese G, De Petrocellis L, Di Marzo V, Appendino G. 2012. Sesquiterpenoids from common ragweed (Ambrosia artemisiifolia L.), an onvasive biological polluter. Eur J Org Chem. 2012(27):5162–5170.

Tantaquidgeon G. 1942. A study of Delaware Indian medicine practice and folk beliefs. Harrisburg (PA): Pennsylvania Historical Commission.

Vazquez-Santillan K, Melendez-Zaigla J, Jimenez-Hernandez L, Martinez-Ruiz G, Maldonado V. 2015. NF-κB signaling in cancer stem cells: a promising therapeutic target? Cell Oncol. 38(5):327–339.

Wopfner N, Jahn-Schmid B, Schmidt G, Christ T, Hubinger G, Briza P, Radauer C, Bohle B, Vogel L, Ebner C, et al. 2009. The alpha and beta subchain of Amb a 1, the major ragweed-pollen allergen show divergent reactivity at the IgE and T-cell level. Mol Immunol. 46(10):2090–2097.