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In vitro cytotoxic activity of abietane diterpenes from *Peltodon longipes* as well as *Salvia miltiorrhiza* and *Salvia sahendica*

M. Fronza [a], R. Murillo [b], S. Ślusarczyk [c,f], M. Adams [c], M. Hamburger [c], B. Heinzmann [d], S. Laufer [e], I. Merfort [a,*]

[a] Department of Pharmaceutical Biology and Biotechnology, University Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany
[b] Escuela de Quimica and CIPRONA, Universidad de Costa Rica, San José, Costa Rica
[c] Department of Pharmaceutical Sciences, Division of Pharmaceutical Biology, University of Basel, Switzerland
[d] Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, Brazil
[e] Department of Pharmaceutical/Medicinal Chemistry, University Tübingen, Germany
[f] Department of Pharmaceutical Biology and Botany, Medical University of Wroclaw, Wroclaw, Poland

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ABSTRACT

Phytochemical investigations of the *n*-hexane extract from the roots of *Peltodon longipes* (Lamiaceae) resulted in the isolation of 12 known abietane diterpenes (**1–12**). Structures were established on the basis of one and two dimensional nuclear magnetic resonance spectroscopic data (^{1}H and ^{13}C, COSY, HSQC and HMBC), electron ionization mass spectrometric analysis (EIMS) as well as comparison with data from literature. These compounds, as well as eight known diterpenes (**13–19**) from *Salvia miltiorrhiza*, and two from *Salvia sahendica* (**20** and **21**) were evaluated for their cytotoxic effects in human pancreatic (MIAPaCa-2) and melanoma (MV-3) tumor cell lines using the MTT assay. Tanshinone IIa (**13**), 7α-acetoxyroyleanone (**1**), 1,2-dihydrotanshinone (**16**) and cryptotanshinone (**14**) had the highest cytotoxic effects in MIAPaCa-2, displaying IC_{50} of 1.9, 4.7, 5.6, and 5.8 μM, respectively. Structure–activity relationships of abietane diterpenoid quinones are discussed.

1. Introduction

Continuing our investigations on Brazilian medicinal plants[1] we here report on a phytochemical and biological study of *Peltodon longipes* (Lamiaceae). The plant is known in Southern Brazil as 'baicuru-amarelo'[2], and preparations from its roots have been used in folk medicine as an antiinflammatory and antiseptic remedy. However, the compounds responsible for these effects are unknown.

Fractionation of the hexane extract resulted in the isolation of 12 known abietane type diterpenes. As some of these had been previously shown to possess cytotoxic activity in various cancer cell lines[3–9], we tested the isolated compounds for cytotoxic effects in the human melanoma cell line MV-3, and in the human pancreatic cancer cell line MIAPaCa-2 using the MTT assay. Melanoma is a malignant tumor which primarily involves the skin and is potentially the most dangerous form of skin tumor with a very high mortality.[10] The incidence of malignant melanoma is increasing faster than any other cancer, and successful systemic chemotherapy is rare. MV-3 cells are highly metastatic in nude mice.[11] Pancreatic cancer shows rapid growth and metastatic distribution. Often chemoresistance is developed during drug therapy, and the survival prognosis is poor.[12] For both types of cancer development of effective drugs is a challenging task, and natural compounds may be an option to identify new leads. Cytotoxic studies revealed that MIAPaCa-2 cells were sensitive to treatment with the diterpenes from *P. longipes*. To gain more insights in structure–activity relationships further abietane diterpenes, mostly with an *ortho*-naphthoquinone skeleton, were included in this study. These compounds had been previously isolated from the roots of two other Lamiaceae species, *Salvia miltiorrhiza* and *Salvia sahendica* roots.

2. Material and methods

2.1. General experimental procedures

Nuclear Magnetic Resonance (NMR) spectra were recorded in $CDCl_3$ on a Bruker DRX 400 MHz instrument (Bruker, Bremen, Germany) at 400 MHz (^{1}H) and 100 MHz (^{13}C). MS data were taken with the following instruments: EI-MS, TSQ 700 mass spectrometer

* Corresponding author. Tel.: +49 761 203 8373; fax: +49 761 203 8383.
E-mail address: irmgard.merfort@pharmazie.uni-freiburg.de (I. Merfort).

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(Thermo Fisher, Waltham, U.S.); ESI-MS, LCQ-Advantage mass spectrometer (Thermo Fisher); HR-EIMS, MAT-95XL double-focusing magnetic field mass spectrometer (Thermo Fisher). Open column chromatography, was carried out with Silica gel 60 (0.063–0.200 mm), and low pressure liquid chromatography with LiChrospher Si 60 (12 μm) (both from Merck). Fractions were monitored by TLC (Silica gel 60 F 254, Merck), and detection was under daylight, under 254 and 366 nm lamps, and after staining with anisaldehyde-H_2SO_4 followed by heating at 110 °C. Sample application in TLC was carried out with a TLC sample applicator (Automatic TLC Sampler 4, CAMAG, Muttenz, Switzerland). HPLC analyses of *P. longipes* and its isolated compounds were carried out on a HP–1090 system (Hewlett–Packard, Palo Alto, CA), equipped with autosampler and photodiode array-detector. A Phenomenex Luna RP-18 column (3 μm; 4.6 × 150 mm) was eluted with mobile phases A (H_2O–ACN, 95:5) and B (ACN–H_2O, 95:5), both containing 0.1% HCOOH. The gradient profile was as follows: linear gradient 60 → 100% of B in 10 min, 100% B until 20 min, and re-equilibration of the column from 20 to 25 min with 60% of B. The flow rate was 0.8 ml/min, and detection at λ 275 nm was used for monitoring. Sample injection was 10 μl.

HPLC separations of *S. miltiorrhiza* were carried out with an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column thermostat and photodiode array (PDA) detector (all Agilent, Waldbronn, Germany). A Gilson 215 liquid handler with Gilson 819 injection module and 50 μl loop was used as autosampler (Gilson; Mettmenstetten, Switzerland). A SunFire RP-18 column (3.5 μm, 3.0 × 150 mm; Waters, Eschborn, Germany) column was eluted with mobile phases A (H_2O) and B (ACN), both containing 0.1% HCOOH. The gradient profile was as follows: linear gradient 50 → 100% B in 30 min, maintaining 100% B for 5 min before re-equilibration. The flow rate was 0.5 ml/min, and detection at λ 300 nm was used for monitoring. 35 μl of a solution of 350 μg/ml extract in DMSO were injected.

2.2. Plant material

The roots of *P. longipes* A. St. Hill. ex Benth. were collected in Santa Maria, South Brazil, in December 2007. Identification was done by the botanist Dr. Gilberto Dolejal Zanetti, Department of Industrial Pharmacy at the Federal University of Santa Maria, Brazil. A voucher specimen (SMDB 12333) has been deposited in the herbarium of the Department of Biology at the same university.

Roots of cultivated *Salvia miltiorrhiza* were kindly provided by Dr. U. Bomme, Bavarian State Institute for Agriculture, Weihenstephan, in July of 2005. Isolation of tanshinone diterpenes has been previously described.[13] A voucher specimen (accession number MTS219) has been deposited in the herbarium of the Division of Pharmaceutical Biology, University of Basel, Switzerland.

The roots of *Salvia sahendica* were collected from the plants natural habitat in Tabriz, Northwestern Iran, at an altitude of 1400 m. Identification of plant material was confirmed by Dr. Ali Sonboli at the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. A voucher specimen has been deposited (accession number MPH-848). Isolation and identification of compounds **20** and **21** has been reported by Jassbi et al.[14]

2.3. Extraction and isolation of diterpenes from *Peltodon longipes*

The air-dried and ground roots (553 g) were exhaustively extracted with *n*-hexane (500 ml) using a Soxhlet apparatus. The solvent was removed under vacuum at 40 °C. After lyophilization a crude *n*-hexane extract (27 g) was obtained. The extract was suspended in 500 ml of MeOH and filtered. Residue (5.2 g) and soluble portion (18.5 g) were stored separately. An aliquot (3.0 g) of the soluble portion was dissolved in EtOAc, adsorbed onto Silica gel (3.0 g), dried and subjected to open column chromatography on Silica gel 60 (150 g, 3.0 × 50 cm). The column was eluted with a *n*-hexane/EtOAc step gradient (starting with 100% *n*-hexane to 100% EtOAc), at a flow rate of 1 ml/min. Fractions of 10 ml were collected, screened by TLC and HPLC, and combined on the basis of similar patterns to 15 fractions (F1-F15). Further separation was carried out on a Silica gel column (LiChrospher® Si 60, 12 μm, 1.5 cm × 40), mixtures of petroleum ether:CH_2Cl_2:EtOAc as eluent and flow rate 0.7 ml/min. F1 afforded **3** (42.8 mg), **5** (12.1 mg), **7** (6.2 mg), and **9** (11.2 mg). F11 yielded **8** (10.1 mg) and **6** (17.5 mg). F8, F13 and F14 gave **2** (65.5 mg), **10** (11.9 mg) and **4** (10.5 mg), respectively. The residue (3 g) was fractionated by open column chromatography on Silica gel 60 (150 g, 3.0 × 50 cm), CH_2Cl_2:MeOH:EtOAc (95:2.5:2.5) as eluent and flow rate 1 ml/min. Four fractions (RF1–RF4) were afforded. RF3 yielded **1** (686.8 mg). RF2 was re-chromatographed on Silica gel (LiChrospher® Si 60, 12 μm, 1.5 cm × 40) by low pressure liquid chromatographic employing petroleum ether:CH_2Cl_2:EtOAc (9.0:0.5:0.5) as eluent, flow rate 0.7 ml/min, to give six sub-fractions (RF2.SF1–RF2.SF6). **12** (5.3 mg) was isolated from RF2.SF1. RF2.SF4 and RF2.SF5 were combined and re-chromatographed yielding **11** (4 mg).

2.4. Cell culture

The human pancreatic cancer cell line (MIAPaCa-2) was obtained from the American Type Culture Collection (ATCC number CRL-1420™, Manassas, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. The human melanoma cancer cell line (MV-3) was a gift from Dr. G.P. van Muijen (Dept. of Pathology, Nijmegen, Netherlands) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2 (all Gibco-BRL, Netherlands).

2.5. Cytotoxicity assay

The MTT assay was carried out according to the method described by Mosmann.[15] Briefly, cells were plated in 96-well flat-bottomed tissue culture plates with 11000–12000 cells per well in 150 μl culture media followed by overnight incubation at 37 °C (5% CO_2 and 95% air) to allow cell attachment to the wells. Cells were incubated for 24 h in the presence or absence of 50 μl of increasing concentrations of the plant extract or pure compounds dissolved in culture medium and DMSO. Camptothecin was used as positive control. Control cells were treated with the highest concentration of DMSO (0.1%) as vehicle control. Then, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (2.5 mg/ml in PBS:Medium (1:2)) was added per well, and the plate incubated for 2 h to allow reaction of MTT by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the formazan crystals formed were dissolved by the addition of 200 μl of extraction solution buffer (20% SDS, 50% DMF). After 12–15 h, absorbance of purple formazan, proportional to the number of viable cells, was measured at 595 nm using a microplate reader (Bio-Rad, Japan).

3. Results and discussion

Fractionation of the *n*-hexane extract from the roots of *Peltodon longipes* by open column chromatography and low pressure liquid chromatography on Silica gel afforded twelve known abietane type

diterpenes. They were identified on the basis of their mass spectra, 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HSQC and HMBC) experiments as 7α-acetoxyroyleanone (**1**)[16,17], 7α-hydroxyroyleanone (syn. horminone) (**2**)[16–18], royleanone (**3**)[16,17,19], 7-ketoroyleanone (**4**)[20], 7α-ethoxyroyleanone (**5**)[21], iguestol (**6**)[22], deoxyneocryptotanshinone (**7**)[23], 12-hydroxy-11-metoxyabieta-8,11,13-trien-7-one (**8**)[24], inuroyleanol (**9**)[22], sugiol (**10**)[16,19,25], cryptojaponol (**11**)[16], and orthosiphonol (**12**)[26] (for structures see Fig. 1).

HPLC analysis of the hexane extract of *P. longipes* was performed, and all isolated diterpenes were assigned to their respective peak in the chromatogram (Fig. 2). Quantification using a calibration curve with the respective isolated compound revealed that 7α-acetoxyroyleanone (**1**) was the main diterpene in the hexane extract with a concentration of 23.4%.

All isolated compounds belong to the abietane diterpenes which are characteristic secondary metabolites of the Lamiaceae family.[8,20,23,25,27] Diterpenes **1–5** and **7** contained a *para*-naphthoquinone moiety which has been repeatedly linked to cytotoxic and antiproliferative activity against human cancer cell lines.[8,9,28]

We tested the extract as well as the isolated compounds in the MIAPaCa-2 and MV-3 cancer cell lines (Table 1). Diterpenes **1**, **2**, **4**, **6**, **8**, and **10** exhibited cytotoxic activity in both cell lines. **1** was the most active with an IC_{50} value of 4.7 μM in MIAPaCa-2 cells and of 7.4 μM in MV-3 cells. Compound **3** only showed moderate activity in the pancreatic cell line, whereas **5**, **7**, **9**, **11**, and **12** had an IC_{50} >100 μM in both cell lines. MIAPaCa-2 cells were mostly more sensitive to the compounds. Interestingly, the *para*-naphthoquinone skeleton was not a prerequisite for cytotoxic effects, as compound **7** was only weakly cytotoxic, whereas the non-quinoidal compound **6** was moderately active. Camptothecin (CPT) was used as a positive control.

Abietane diterpenes with an *ortho*-naphthoquinone moiety are also found in Lamiaceae species.[8,9,28] To compare the effect of *ortho* and *para*-quinone moieties on cytotoxicity we included six 20-nor-abietanes (**13–18**) and one secoabietane diterpene (**21**) with an *ortho*-naphthoquinone structure, and two diterpenes lacking a quinone structure (**19–20**). These compounds had been previously isolated from roots of *Salvia miltiorrhiza*[13] and *S. sahendica* (compounds **20** and **21**).[14] We focused in our study on MIAPaCa-2 cells. Tanshinone IIa (**13**), an abietane with a furan ring, exhibited the highest cytotoxicity in MIAPaCa-2 cells (IC_{50} 1.9 μM). Compounds bearing a dihydrofuran ring (**14**) or additional double bonds in ring A (**15** and **16**) were less cytotoxic. An isopropyl moiety instead of the heterocyclic D-ring further lowered activity (IC_{50} of 22.5 and 29.9 μM for **17** and **18**, respectively). The monohydroxy derivative **20** gave a similar IC_{50} value of 25.9 μM. Cytotoxic activity dramatically decreased with the dihydroxy derivative **19** which additionally has a carbonyl group (IC_{50} 66.3 μM). Interestingly, the secoabietane **21** had an IC_{50} of 10.2 μM, although missing an intact ring A. Considering only the abietane diterpenes with an *ortho*-quinone moiety our results confirm the recent SAR proposal of Wang et al.[9] who suggested an *ortho*-quinone moiety in ring C, an intact ring D, and a relatively planar structure as relevant structural features for cytotoxicity of these types of diterpenes. However, as

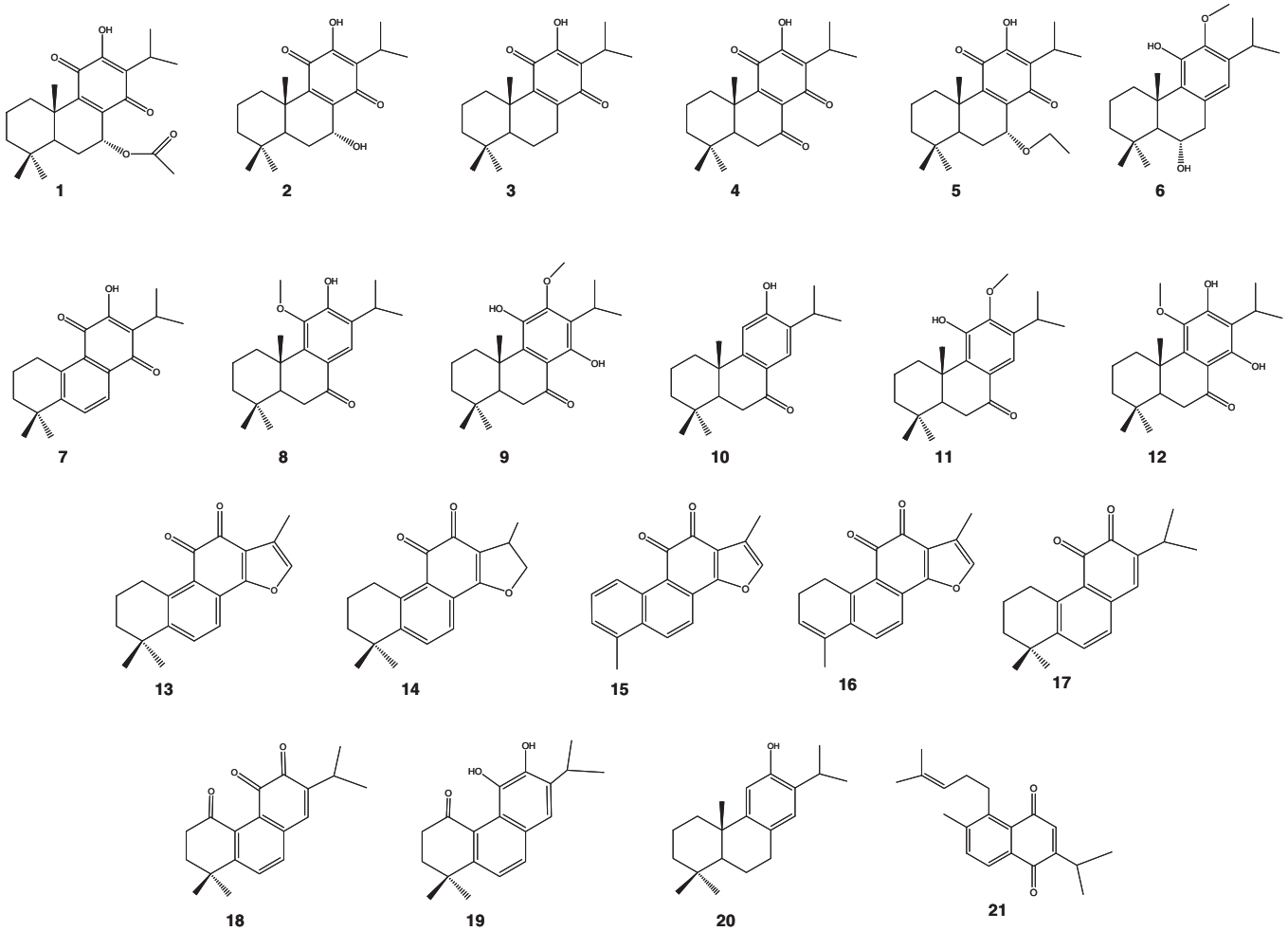

Figure 1. Chemical structures of the diterpenes isolated from *Peltodon longipes* (**1–12**), *Salvia miltiorrhiza* (**13–19**) and *Salvia sahendica* (**20** and **21**).

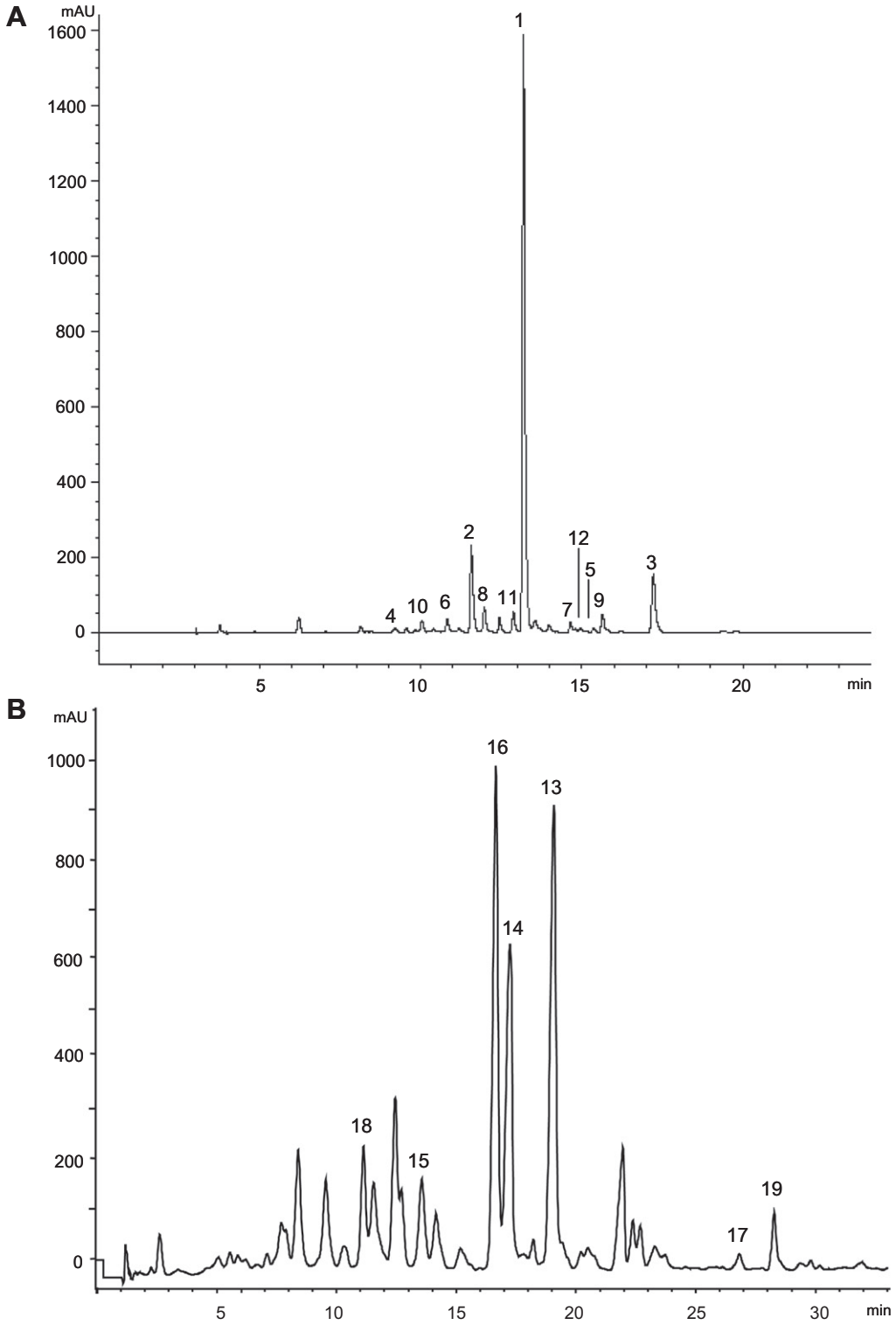

Figure 2. HPLC chromatogram of the *n*-hexane extract from *Peltodon longipes* (injection: 10 µl (=5 µg extract), concentrations of the main compound: **1**: 24.3% (A) and of the *n*-hexane extract of *Salvia miltiorrhiza* (injection: 35 µl = 350 µg extract) (B). Further HPLC conditions are given in the Experimental Section.

the *para*-quinone derivative **1** also exhibited a strong cytotoxic activity (IC_{50} 4.7 µM), an *ortho*-quinone in ring C and the presence of ring D are not the only structural requirements for a high cytotoxicity. Compound **1** was already shown by Araujo et al.[3] to possess a high cytotoxic effect in five other cancer cell lines. Notably, despite the fact that *ortho*-quinones were generally more active than the *para*-quinones, activities of the crude extracts of *P. longipes* and *S. miltiorrhiza* were comparable (IC_{50} 1.3 µg/ml and 1.8 µg/ml, respectively).

Summarizing our study with 21 abietane and secoabietane derivatives, no straightforward structural prerequisites for strong cytotoxic activity can be described. However, one may have to take into account that the underlying mechanisms for cytotoxicity of the investigated diterpenes may differ. It has been suggested

Table 1
Cytotoxic activity of the diterpenes in MIAPaCa-2 and MV-3 cancer cell lines. IC_{50} values and 95% confidence intervals from three independent experiments are given. Camptothecin was used as positive control.

Compound/Extract	Cell line IC_{50} [μM]	
	MIAPaCa-2	MV-3
Peltodon longipes - extract*	1.3* (1.2–1.4)	2.9 (2.8–3.1)
7α-Acetoxyroyleanone (**1**)	4.7 (4.4–5.1)	7.4 (4.9–11.1)
Horminone (**2**)	27.5 (25.5–29.7)	16.7 (14.7–18.9)
Royleanone (**3**)	32.5 (29.2–36.0)	>80
7-Ketoroyleanone (**4**)	30.1 (28.2–31.2)	65.8 (61.0–70.8)
7α-Ethoxyroyleanone (**5**)	>100	>80
Iguestol (**6**)	41.3 (34.5–46.8)	65.9 (60.4–71.9)
Deoxyneocryptotanshinone (**7**)	>100	>150
12-Hydroxy-11-methoxyabieta-8,11,13-trien-7-one (**8**)	34.9 (29.7–41.0)	32.3 (30.4–34.3)
Inuroyleanol (**9**)	>80	>120
Sugiol (**10**)	17.9 (15.6–20.6)	34.1 (30.0–39.1)
Cryptojapanol (**11**)	>100	>80
Orthosiphonol (**12**)	>100	>80
Salvia miltiorrhiza - extract*	1.8* (1.5–2.3)	nd
Tanshinone IIa (**13**)	1.9 (1.6–2.3)	nd
Cryptotanshinone (**14**)	5.8 (4.9–6.9)	nd
Tanshinone I (**15**)	10.5 (9.1–12.1)	nd
1,2-Dihydrotanshinone (**16**)	5.6 (4.7–6.7)	nd
Miltirone (**17**)	22.5 (21.7–23.5)	nd
1-Oxomiltirone (**18**)	29.9 (26.4–33.8)	nd
Miltiodiol (**19**)	66.3 (54.9–80.1)	nd
Ferruginol (**20**)	25.9 (23.5–28.7)	nd
Sahandinone (**21**)	10.18 (9.08–11.41)	nd
Camptothecin	0.4 (0.3–0.5)	nd

* IC_{50} expressed in μg/ml; nd: not determined

that biological membranes could be a primary target, and that the protonophoric activity could contribute to cytotoxicity.[29] On the other hand, tanshinone IIa (**13**) was recently shown to induce mitochondria dependent apoptosis in association with an inhibition of the phosphoinositide 3-kinase/AKT pathway.[30] For 1,2-dihydrotanshinone (**16**) inhibition of topoisomerase I has been shown[31], whereas 7α-acetoxyroyleanone (**1**), horminone (**2**) and royleanone (**3**) exhibited DNA damaging activity.[6] Therefore, further studies are necessary for a deeper understanding of the molecular targets of these abietane diterpenoids to identify the most promising compound with potential for further optimization towards an antiproliferative agent before selecting a lead for the development of drugs treating tumors, such as pancreatic cancer or melanomas.

4. Conclusions

In summary, the first phytochemical study of *P. longipes* resulted in the isolation of 12 abietane diterpenes. These secondary metabolites may explain the traditional use of *P. longipes* preparations. Moreover, we were able to extend current knowledge on structure–activity relationships in abietanes by inclusion of related compounds with an *ortho*-quinone moiety in the test compound series.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.067. These data include MOL files and InChiKeys of the most important compounds described in this article.

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