

Cytotoxic Activity of Royleanone Diterpenes from *Plectranthus madagascariensis* Benth

Diogo Matias,^{†,‡} Marisa Nicolai,[†] Lucília Saraiva,[§] Rute Pinheiro,[§] Célia Faustino,[⊥] Ana Diaz Lanza,[‡] Catarina Pinto Reis,^{†,⊥} Tijana Stankovic,^{||} Jelena Dinic,^{||} Milica Pesic,^{||} and Patrícia Rijo^{*,†,⊥}

[†]Research Center for Biosciences & Health Technologies (CBIOS), Universidade Lusófona de Humanidades e Tecnologias, Campo Grande 376, 1749-024 Lisboa, Portugal

[‡]Department of Biomedical Sciences, Faculty of Pharmacy, University of Alcalá, Campus Universitario, 28871 Alcalá de Henares, Spain

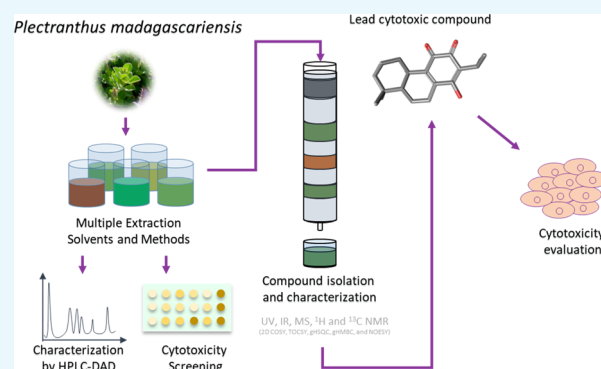
[§]LAQV/REQUIMTE, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

^{||}Institute for Biological Research “Siniša Stanković”, University of Belgrade, Despota Stefana 142, 11060 Belgrade, Serbia

[⊥]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Avenida Professor Gama Pinto, 1649-003 Lisboa, Portugal

Supporting Information

ABSTRACT: Cytotoxicity screenings have identified *Plectranthus* plants as potential sources of antitumor lead compounds. In this work, several extracts from *Plectranthus madagascariensis* were prepared using different solvents (acetone, methanol, and supercritical CO₂) and extraction techniques (maceration, ultrasound-assisted, and supercritical fluid extraction), and their chemical composition was detailed using high-performance liquid chromatography with a diode array detector. The cytotoxic activity of the major compounds identified, namely, rosmarinic acid (**1**) and abietane diterpenes 7 α ,6 β -dihydroxyroyleanone (**2**), 7 α -formyloxy-6 β -hydroxyroyleanone (**3**), 7 α -acetoxy-6 β -hydroxyroyleanone (**4**), and coleon U (**5**), was evaluated in a battery of human cancer cell lines, including breast (MDA-MB-231, MCF-7), colon (HCT116), and lung (NCI-H460, NCI-H460/R) cancer, and also in healthy lung (MCR-5) cells. Royleanone (**3**) was isolated for the first time from *P. madagascariensis*, and its full spectroscopic characterization (proton and carbon nuclear magnetic resonance) was accomplished. A high selectivity for lung cancer cells was observed for royleanones (**2**, **4**) with selectivity indexes of 4.3 and 3.2, respectively. The observed results combined with literature data allowed the establishment of important structure–activity relationships for substituted royleanone abietanes, such as the requirement for an electron-donating group at positions 6 and/or 7 in the abietane skeleton, and an improved cytotoxic effect for substituents with log *P* values between 2 and 5.



INTRODUCTION

Plectranthus species have been traditionally used for a wide range of complaints,¹ mainly of the respiratory system, gastrointestinal tract, nervous system, skin disorders, infections, pain, and different types of cancer due to their medicinal properties, which include antimicrobial,^{2–5} antioxidant,^{3,6} anti-inflammatory,^{7,8} and cytotoxic^{7–11} activities. Several cytotoxic activity studies have been performed on *Plectranthus* spp. Cytotoxicity screening of South African medicinal plants traditionally used in the treatment or prevention of cancer¹² revealed that the extracts of *Plectranthus ciliatus* and *Plectranthus barbatus* inhibited the growth of both sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 leukemia cells. Similarly, in another in vitro screening using the RBL-2H3 cell line,⁸ *P. ciliatus* showed higher cytotoxicity

among the acetone extracts of the seven *Plectranthus* species tested.

The organic extracts of *Plectranthus amboinicus* also exhibit relevant cytotoxicity. The ethyl acetate extract inhibited the growth of breast cancer MCF-7 cells,¹³ whereas the ethanol extract suppressed growth and induced apoptosis in the human lung cancer A549 cell line.¹⁴ Moreover, the hydroalcoholic extract from leaves of *P. amboinicus* showed interesting in vivo antitumor effects.⁷ On the other hand, the ethanol extract from aerial parts of *Plectranthus neochilus* has shown cytotoxic effects in the *Artemia salina* model,¹⁵ and more recently, a hexane

Received: February 23, 2019

Accepted: April 18, 2019

Published: May 2, 2019

Table 1. Preparation, Yields, and Component Quantification of *P. madagascariensis* Extracts

extract	solvent	method ^a	extraction yield (mg/g)	component yield in extract (mg/g) ^b				
				(1)	(2)	(3)	(4)	(5)
E1	acetone	UAE	1.51	29.8	4.62	1.64	1.04	15.5
E2	acetone	ME	1.45	17.5	3.19	6.74	1.21	5.77
E3	methanol	UAE	6.56	4.60	4.20	0.81	0.77	t
E4	methanol	ME	12.0	26.4	1.05	0.24	t	t
E5	scCO ₂	SCFE	1.31	17.8	4.98	0.20	0.84	n/d
E6	acetone	R-SCFE	2.95	50.5	0.33	0.17	n/d	n/d

^aUAE, ultrasound-assisted extraction; ME, maceration extraction; SCFE, supercritical fluid extraction; R-SCFE, re-extraction of SCFE remaining plant material. ^bt, traces. n/d not detected. The extraction yield is expressed in mg of extract per g of plant dry material. The component yield is expressed in mg of component per g of plant dry material.

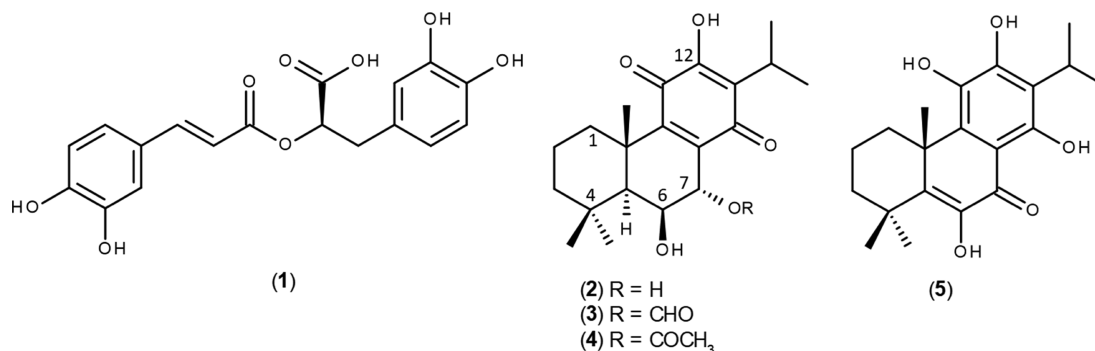


Figure 1. Chemical structure of the major components in *P. madagascariensis* extracts: rosmarinic acid (1), 6β,7α-dihydroxyroyleanone (2), 7α-formyloxy-6β-hydroxyroyleanone (3), 7α-acetoxy-6β-hydroxyroyleanone (4), and coleon U (5).

extract showed growth inhibitory effects in cell carcinoma cell lines.¹⁶

The promising results from the screening of *Plectranthus* extracts followed by the bio-guided isolation of the active compounds led to the elucidation of some promising anticancer lead compounds, namely, labdane and abietane diterpenes. The labdane diterpene forskolin, isolated from the roots of *P. barbatus*, was one of the first compounds isolated from *Plectranthus* species with promising anticancer activity.¹⁷ Furthermore, forskolin was shown to be a potent inhibitor of the growth of colon cancer cells with induction of cycle arrest at the G₁ phase and further apoptosis.¹⁸

Abietane diterpenes of the royleanone and coleon type commonly found in the *Plectranthus* genus, as well as other abietanoid quinone methides, such as Parvifloron D isolated from *Plectranthus ecklonii*,⁹ have been described as potent antiproliferative and/or cytotoxic agents.^{9–11,19–21}

Previous cytotoxicity activity screening performed by our group from *Plectranthus madagascariensis*, *P. neochilus*, and *Plectranthus porcatus* revealed promising cytotoxic activity of the *P. madagascariensis* extract obtained by maceration in acetone and in the MDA-MB-231 breast cancer cell line,²⁴ encouraging further research. In this study, the chemical composition of several *P. madagascariensis* extracts obtained using different organic solvents and extraction methodologies was detailed using high-performance liquid chromatography with a diode array detector (HPLC-DAD) and complementary spectroscopic methodologies, and the major compounds were identified and quantified. The cytotoxic effects of the pure compounds were evaluated in human breast, lung, and colon cancer cell lines, and relevant structure–activity relationships were disclosed for the royleanone-type abietane diterpenes.

RESULTS AND DISCUSSION

Preparation of *P. madagascariensis* Extracts. Several extracts from *P. madagascariensis* have been prepared using different solvents (acetone, methanol, and supercritical CO₂) and extraction techniques (maceration, ultrasound-assisted, and supercritical fluid extraction), resulting in different extractive yields (Table 1).

Higher extractive yields were obtained using methanol as the solvent, which can be due to the ability of alcohol to disrupt the plant cell wall favoring the diffusion of secondary metabolites to the bulk extract. Moreover, considering the relative polarity of the extraction solvents in terms of dielectric constant (ϵ), a trend was observed for the more polar solvents to achieve higher extraction yields ($\epsilon_{\text{methanol}} > \epsilon_{\text{acetone}} \gg \epsilon_{\text{scCO}_2}$). The supercritical fluid extraction (SCFE) method using scCO₂ was the least efficient extraction process with the lower yield. Because of this low extraction efficiency, a re-extraction of the remaining plant material with acetone was performed to extract the remaining secondary metabolites, which resulted in a higher extraction yield (E6, Table 1) when compared to SCFE alone.

HPLC-DAD Extract Profiling. The identification and quantification of major compounds (rosmarinic acid (1), 6β,7α-dihydroxyroyleanone (2), 7α-formyloxy-6β-hydroxyroyleanone (3), 7α-acetoxy-6β-hydroxyroyleanone (4), and coleon U (5); Figure 1) in *P. madagascariensis* extracts were performed using HPLC-DAD and complementary spectroscopic methodologies.

Previous phytochemistry studies on the *Plectranthus* genus revealed the presence of polyphenols and diterpenes as the most frequent secondary metabolites.^{3,6,32} These compounds have characteristic absorption patterns in the UV spectral region due to the presence of conjugated carbonyl groups (270

nm), aromatic rings (280 nm), and phenolic groups (330 nm); thus, analytical measurements were performed at these three wavelengths. HPLC representative chromatograms of *P. madagascariensis* extracts are available at the Supporting Information (Figure S1). For each extract, the major peaks were identified by comparing the retention time and UV-vis spectrum overlay (Figure S2, Supporting Information) by co-elution with pure standards. The peak eluted at 10.47 min, which was present in all extracts, was positively identified as rosmarinic acid (1, Figure 1) after co-elution with a commercial standard (Figure S2, Supporting Information). This polyphenol, found in several *Plectranthus* species, had been previously identified in *P. madagascariensis*.^{5,33}

The peaks obtained at the average retention times of 17.80, 19.40, and 19.80 min possessed the typical royleanone-type abietane UV spectra²⁵ (Figure S1, Supporting Information) with an absorption maximum at 272 nm and a secondary broad maximum between 300 and 500 nm, in accordance with other phytochemical studies of *Plectranthus* species.³² The peaks at 17.80 and 19.80 min were identified as 6 β ,7 α -dihydroxyroyleanone (2, Figure 1) and 7 α -acetoxy-6 β -hydroxyroyleanone (4, Figure 1), respectively, after co-elution of the extracts with authentic samples previously obtained from *Plectranthus* spp.² The peak at 19.40 min was identified as 7 α -formyloxy-6 β -hydroxyroyleanone (3, Figure 1), isolated from the acetone extract prepared by UAE, after co-elution with the purified compound. A major peak eluted at 21.08 min showing a UV spectrum (Figure S1, Supporting Information) comparable to that of coleon U (5, Figure 1),²³ a diterpene often found in *Plectranthus* species, which was confirmed after co-elution of the extracts with an authentic sample.² This compound was reported to have some intrinsic instability, being easily converted to an oxidized form, coleon U-quinone (6, Figure 2).^{23,25} This degradation product was also detected in some *P.*

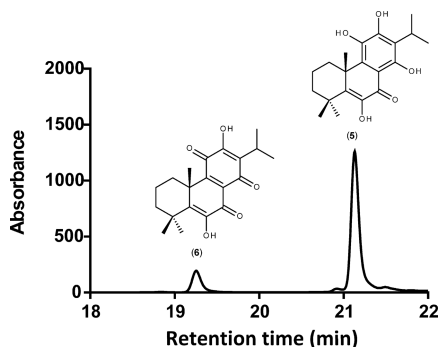


Figure 2. HPLC chromatogram showing the decomposition of coleon U (5) to coleon U quinone (6).

madagascariensis extracts as a trace at a retention time of 19.19 min. The periodic analysis of an authentic sample of (5) revealed the gradual increase of the peak at 19.19 min and concomitant reduction of the peak at 21.08 min, which represents a gradual conversion of the compound (5) into its oxidized form (6, Figure 2).

Isolation and Spectroscopic Structure Elucidation of 7 α -Formyloxy-6 β -hydroxyroyleanone (3). The compound (3) was isolated from the acetone extract of *P. madagascariensis* (480 mg of dried residue) prepared by UAE following column chromatography using hexane/ethyl acetate 95:5 (v/v) as an eluent and purified using preparative chromatography through repeated elution with hexane/ethyl acetate 75:25 (v/v),

yielding 6.1 mg (1.27% (w/w) yield) of yellow needles after recrystallization from methanol. The compound was fully characterized using NMR spectroscopy (¹H and ¹³C NMR spectra available in the Supporting Information, Figures S3 and S4, respectively).

The ¹H NMR spectrum of (3) (Figure S3, Supporting Information) was very similar to that of royleanone (4).²⁹ The main differences were due to the absence of the singlet signal at δ 2.02 ppm corresponding to the methyl protons of the acetoxy group at C7 in (4) and the presence of a distinct doublet at δ 8.05 ppm (J = 1.2 Hz), which might correspond to an acidic proton from a secondary formyloxy group.³⁰ This observation suggested the substitution of the 7 α -acetoxy group of (4) by 7 α -formyloxy in (3), which was confirmed by the literature³⁰ and extensive NMR studies (¹H and ¹³C NMR, COSY, HMBC, and HMQC; Table 2). Thus, the compound

Table 2. NMR Spectroscopic Data (400 MHz, CDCl₃) for 7 α -Formyloxy-6 β -hydroxyroyleanone (3)

position	δ_C , type	δ_H (J in Hz)	HMBC
1	38.49, CH ₂	1.19, s; 2.64, d (12.9)	
2	19.09, CH ₂	1.50, m; 1.85, d (4.5)	
3	42.45, CH ₂	1.20, 1.50, m	
4	33.83, C		
5	49.80, CH	1.38, s	10
6 α	67.25, CH	4.37, m	
7 β	68.42, CH	5.80, m	
8	136.45, C		
9	150.38, C		
10	38.79, C		
11	183.34, C		
12	151.13, C		
13	124.35, C		
14	183.34, C		
15	24.34, CH	3.17, qi (14.1; 7.1)	13
16	33.85, CH ₃	1.24, d (7.1)	
17	20.01, CH ₃	1.21, d (7.1)	13
18	33.57, CH ₃	0.96, s	3, 5, 19
19	23.97, CH ₃	1.25, s	3, 5, 18
20	21.71, CH ₃	1.62, s	1, 5
21	159.64, CH	8.04, d (1.2)	
6-OH (1)		2.31, t (7.5)	
12-OH (2)		7.20, m (3.1)	

(3) was identified as 7 α -formyloxy-6 β -hydroxyroyleanone, which has been previously isolated from *Plectranthus hadiensis*³¹ and *Plectranthus myrianthus*.²⁵ To our knowledge, this was the first isolation of (3) from *P. madagascariensis* and its first full ¹³C NMR characterization.

Kubinová et al.⁵ have studied the methanol extract of *P. madagascariensis*, which resulted in the identification of rosmarinic acid (1), 6 β ,7 α -dihydroxyroyleanone (2), 7 α -acetoxy-6 β -hydroxyroyleanone (4), and coleon U-quinone (6) as the major components of this extract. However, the full spectroscopic elucidation of these compounds was not accomplished by the authors.

The quantification of the major compounds in *P. madagascariensis* extracts was also performed based on the validated calibration curves for main polyphenol rosmarinic acid (1) and main abietane diterpenes 6 β ,7 α -dihydroxyroyleanone (2), 7 α -formyloxy-6 β -hydroxyroyleanone (3), 7 α -acetoxy-6 β -hydroxyroyleanone (4), and coleon U (5). Linear

responses and high sensitivity were obtained for all compounds (Table S1, Supporting Information). The component yield in each extract has been listed in Table 1.

7 α -acetoxy-6 β -hydroxyroyleanone (4) obtained from *Plectranthus grandidentatus* is described to show strong in vitro antiproliferative activity against several human cancer cell lines,¹¹ whereas taxodione from *P. barbatus* and their semisynthetic derivatives were highly cytotoxic toward human leukemia cells.¹⁹ Growth inhibition triggered by these compounds was caused by induction of apoptosis, whereas cell death was associated with the release of mitochondrial proteins due to mitochondrial membrane depolarization.¹⁹ Additionally, 7 α -acetoxy-6 β -hydroxyroyleanone (4) was identified as the main compound that contributed to the cytotoxic activity of the chloroform extract of *P. amboinicus* against breast cancer MCF-7 cells, using an HPLC-based metabolomics approach.²²

Rosmarinic acid (1) was the most abundant secondary metabolite present in all extracts, with yields ranging from 4.60 to 50.5 mg/g (Table 1). This finding is in agreement with the described abundance of this polyphenol in other *Plectranthus* species.^{6,32} Coleon U (5) was diterpene quantified in higher yields, although high yields of royleanone-type diterpenes (2–4) were also found in all extracts, particularly in the acetone ones (E1 and E2, Table 1).

Coleon U has been isolated from several *Plectranthus* species, such as *Plectranthus forsteri*,²³ *P. grandidentatus*,^{10,11} *P. madagascariensis*²⁴ and *P. myrianthus*,²⁵ exhibits potent cytotoxic effects transversal to several human cancer cell lines, including breast,^{11,19} lung,¹¹ leukemia,^{10,19,26} and melanoma.^{11,19} Moreover, coleon U has been described as a potent and selective activator of the proapoptotic protein kinases C- δ (PKC δ) and C- ϵ (PKC ϵ), which can be on the basis of its reported antitumor action.^{27,28} However, coleon U is easily degraded to its oxidized form, coleon U-quinone. This metabolite has been isolated from *Plectranthus xanthanthus* along with coleon U 11-acetate, and both showed cytotoxic effects.^{23,26}

Cytotoxicity Evaluation. The cytotoxic effects of *P. madagascariensis* extracts have been previously evaluated in the MDA-MB-231 cancer cell line by our group,²⁴ and the isolated abietane diterpenes most likely contribute to the cytotoxicity of *P. madagascariensis* extracts. The extract obtained by maceration with acetone (E2), which had the highest combinatory yield of royleanone abietanes (Table 1), was the most cytotoxic.²⁴ The cytotoxic activities of the royleanone diterpenes, that is, against breast cancer cell lines, have been previously reported.^{11,19} Additionally, coleon U (5) has been described in the literature as a potent cytotoxic agent, being active against breast,^{11,19} leukemia,^{10,19,26} and melanoma^{11,19} cancer cell lines. However, the acetone extract (E1) prepared by UAE and previously analyzed,²⁴ which had the highest yield of coleon U (5) and also the highest combinatory yield of abietane diterpenes, was not the most cytotoxic. This can be due to its higher amount of rosmarinic acid (1) because the most cytotoxic extract (E2) had a lower yield of this polyphenol compared to the other extracts (Table 1). Rosmarinic acid (1) is known to be a potent antioxidant,⁶ and some studies related the cytotoxic effect of abietane diterpenes possessing conjugated quinone moieties to the induction of radical reactions,^{20,21} which may anticipate a potential antagonistic effect between antioxidant polyphenols and conjugated quinone diterpenes.

The cytotoxic activity of isolated compounds (1–5) from *P. madagascariensis* extracts was assayed in breast cancer (MDA-MB-231 and MCF-7), colon cancer (HCT116), non-small cell lung cancer (NCI-H460), and normal lung bronchial (MCR-5) cell lines (Table 3). Interestingly, in the MCF-7 cell line, the structural modifications carried out in compounds (3–5) significantly increased their antiproliferative effects when compared to that in the compound 2 (Table 3). All abietane diterpenes revealed growth inhibitory effects in most of the cancer cell lines tested. Moreover, royleanones (2, 4) exhibited high selectivity based on the comparison of GI₅₀ for cancer (NCI-H460) and normal lung (MCR-5) cell lines. These royleanones also showed similar growth inhibition of the NCI-H460 cancer cell line and its multidrug-resistant variant (NCI-H460/R), which overexpress the multidrug resistance protein 1 (MDR1 or P-glycoprotein),³⁴ suggesting that compounds (2, 4) are not substrates for such efflux pumps.

On the one hand, the growth of MDA-MB-231 cancer cells was not particularly affected by abietane diterpenes. This cell line is a highly metastatic triple negative breast cancer cell line that does not display estrogenic receptors (ER), progesterone receptors (PR), or human epidermal growth factor receptor 2 (HER2), thus being clinically difficult to target.³⁵ The ER negative cells are known to have a higher expression of PKC classic isoforms when compared to ER positive cell lines.³⁶ The upregulation of the classic isoform PKC α promotes the invasiveness and metastasis formation³⁶ along with increased drug resistance³⁷ in breast cancers. On the other hand, the PKC δ activation supports both prosurvival³⁸ and proapoptotic³⁹ functions in breast cancer cells. Some abietane diterpenes such as coleon U (5) have also been shown to exert proapoptotic effects by the specific activation of PKC- δ and PKC- ϵ .^{27,28} Therefore, in the MDA-MB-231 cell line characterized by an overexpression of classic PKC isoforms in detriment of new PKC isoforms, the preferential mechanism of apoptosis induction by coleon U (and eventually by other abietane diterpenes) through activation of PKC- δ and PKC- ϵ should be less effective, which can explain the lower growth inhibition of this cell line by these compounds.

Some relationships between the PKC overexpression and the drug resistance by MDR1 have been reported. A cellular increase of the MDR1 expression was verified following the treatment with the PKC activator TPA, which was suppressed by the use of a PKC inhibitor (staurosporine).⁴⁰ Furthermore, the use of the PKC inhibitor, bryostatin 1, potentiated the cytotoxic effects of anticancer drugs transported by efflux pumps, such as vincristine, by the reduction of MDR1 expression.⁴¹ Those findings can be especially relevant in the case of abietane diterpenes being not transported by MDR1 for which the PKC activation and the secondary MDR1 overexpression should not increase the cell resistance to such compounds.

Structure–Activity Relationships. The obtained data on abietane diterpene cytotoxicity (Table 3) combined with other studies on the cytotoxicity of this class of compounds (Table 4) allowed the establishment of some structure–activity relationships (SARs) for royleanone-type abietanes. The main difference between royleanones (2) and (4) resides on the polarity of the substituent at 7 α (Figure 1). In this series, a clear tendency was observed for increasing cytotoxicity with the higher lipophilicity of the 7 α substituent. The same tendency was observed between horminone (Figure 3, R₁ = H, R₂ = R₃ = OH) and its more cytotoxic 7 α -acetoxy derivative

Table 3. Concentration of Major Compounds from *P. madagascariensis* Extracts Causing 50% Cell Growth Inhibition ($GI_{50}/\mu M$) of Human Cell Lines^a

compound	sulfurhodamine B (SRB) assay					MTT assay				
	MDA-MB-231	MCF-7	HCT116	NCI-H460	NCI-H460/R	MCR-5	SI			
(1)	>100	nt	nt	>100	>100	>100				
(2)	>100	26.0 ± 0.6	≥50	25 ± 2	25 ± 2	91 ± 13	4.3*			
(3)	>100	7.9 ± 0.88	7.9 ± 1.2	14.9 ± 2.9	nt	nt				
(4)	>100	6.4 ± 0.4 ^{#§}		2.7 ± 0.4	3.1 ± 0.4	8.6 ± 0.4	3.2*			
(5)	46.9	5.5 ± 0.8 ^{#§}		3.0 ± 0.2 [#]	nt	nt				
positive control	DOX 0.072 ± 0.0021	DOX 0.16 ± 0.0018	DOX 0.125 ± 0.0013	PCX 0.0006 ± 0.0001	PCX 0.117 ± 0.013	PCX 0.523 ± 0.001	PCX 872 ^{***}			

^aSelectivity index, SI = $GI_{50}(\text{MCR-5})/GI_{50}(\text{NCI-H460})$. nt, not tested. [#]Previously reported results from our group.¹¹ Doxorubicin (DOX) was used as positive control in MDA-MB-231, MCF-7, and HCT116 cells. Paclitaxel (PCX) was used as positive control in NCI-H460, NCI-H460/R, and MCR-5 cells. Significant selectivity toward cancer cells: * $p < 0.05$, *** $p < 0.001$ (in NCI-H460, NCI-H460/R, and MCR-5 cells). [§]Values significantly different from compound (2), $P < 0.05$.

(Figure 3, $R_1 = \text{H}$, $R_2 = \text{OCOCH}_3$, $R_3 = \text{OH}$).^{42,43} Whether these structural alterations corresponded to an improved fitting on the target or if the cytotoxic activity was instigated by favorable log P for membrane crossing remains to be answered because the cytotoxicity of several diterpenes has been associated with a mechanism involving membrane-disrupting properties.^{3,44}

On the basis of the extensive literature reporting the cytotoxicity on royleanone-type compounds bearing a *p*-benzoquinone moiety in the ring C (Figure 3), additional tendencies could be explored (Table 4).

Burmistrova et al.¹⁹ studied the cytotoxic effects for a series of derivatives of 7 α -acetoxy-6 β -hydroxyroyleanone (4). The overall compounds affect the cell proliferation with an apparently cell-type dependent intensity. By displaying such compounds by their log P value, a strong tendency for higher cytotoxic effects was observed for log P values between 2 and 5.5. Because the “Lipinski rule of five” establishes that log P for an oral bioavailable compound should be under 5, the useful compounds must be considered in the 2–5 range of log P .⁴⁵ The only exception for this trend was royleanone (Figure 3, $R_1 = R_2 = \text{H}$, $R_3 = \text{OH}$) that showed only slight cytotoxic effects in some cell lines.^{19,42,46} This could indicate that the presence of a lipophilic substituent was required at positions 6 and/or 7 for the cytotoxic effects to take place. However, the 6,7-dehydro derivative (Figure 3, $R_1 = R_2 = \text{H}\Delta$,^{6,7} $R_3 = \text{OH}$) has shown some potent cell-type specific cytotoxic effects.^{47–49} Thus, the presence of an electron-donating group at position 6 or 7 seems to be essential for cytotoxic activity.

The cytotoxicity of the studied abietane diterpenes against most of the cancer cell lines tested and the selectivity of the royleanone-type compounds, particularly for non-small lung cancer cells and their multidrug-resistant variant, encourage further studies using this scaffold to develop analogues with potential application as chemopreventive, chemoadjuvant, or chemotherapeutic agents.

EXPERIMENTAL SECTION

General Experimental Procedures. Chemical reagents and standards were from Sigma-Aldrich and used as received. Authentic samples of 7 α ,6 β -dihydroxyroyleanone (2), 7 α -acetoxy-6 β -hydroxyroyleanone (4), and coleon U (5) were obtained from Gaspar-Marques.² Solvents of either analytical or HPLC grade were from Merck. Fetal bovine serum (FBS) was from Gibco, penicillin–streptomycin for the cell culture and an RPMI 1640 culture medium with ultraglutamine were from Lonza, and Dulbecco’s modified Eagle’s medium (DMEM) was from Biowest. Analytical TLC and preparative TLC were performed on precoated silica gel plates from Merck (Kieselgel 60 F254, 0.2 and 0.5 mm). For column chromatography, silica gel 60 (0.063–0.200 mm) from Merck was used. NMR spectra were recorded on a Varian INOVA-400 spectrometer (Varian, Palo Alto, CA, USA) equipped with a 5 mm inverse detection z-gradient probe. The ¹H and ¹³C NMR spectra were acquired at 400 and 100 MHz, respectively, using CDCl₃ as the solvent, and the chemical shifts were reported in parts per million referring to the residual CHCl₃ signal (δ_{H} 7.26 for proton and δ_{C} 77.0 for carbon).

Cell Cultures. The human cell lines MDA-MB-231 (metastatic breast cancer), MCF-7 (estrogen-dependent breast carcinoma), HCT116 (colorectal carcinoma), NCI-H460 (non-small cell lung carcinoma), and MCR-5 (normal

Table 4. Cytotoxicity of Several Royleanone Derivatives against Different Cell Lines According to the Literature^{a,b}

compound features ^c			cell lines																	
R ₁	R ₂	R ₃	log P	breast	CNS	colon	gastric	leukemia/lymphoma	melanoma	papilloma	pancreas	renal	lung	normal	melanoma					
				MCF-7 ^{1,19}	SF-268 ^{1,19}	HCT116 ^{1,19}	AGS ⁴⁶	HL-60 ^{7,48}	P-388 ⁴⁹	U937 ¹⁹	Molt-3 ¹⁹	MEL-1 ¹⁹	UACC-62 ¹¹	MV-3 ⁴²	KB	PaCa-2 ⁴²	TK-10 ¹¹	H460 ⁴⁶	MCR-S ⁴⁶	
OH	OH	OH	0.85	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
OH	OCHO	OH	0.97	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	+
OH	OCOCH ₃	OH	1.08	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	++
OCOCH ₃	OCOCH ₃	OH	1.31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++
OCOCH ₃	OCOCH ₃	OCOCH ₃	1.54	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
H	=O	OH	1.57																	
H	βOH	OH	1.74				-	-	-	-	-	-	-	-	-	-	-	-	-	+
H	OH	OH	1.74				+	+	+	+	+	+	+	+	+	+	+	+	+	+
OCOCH ₃ CH ₃	OCOCH ₃	OH	1.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H	OCOCH ₃	OH	1.97																	
OH	OCH ₃	OH	2.10																	
OH	OCOCH ₃	OCOPh(4-NO ₂)	2.30	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OCO(CH ₂) ₂ CH ₃	OCOCH ₃	OH	2.38	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
HA ⁶⁷	HA ⁶⁷	OH	2.51				-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
H	H	OH	2.83				-	-	-	-	-	-	-	-	-	-	-	-	-	+
OCOCH ₃ CH ₃	OCOCH ₃	OCOCH ₂ CH ₃	2.85	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OH	OCOCH ₃	OCOPh(4-CH ₃)	3.69	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
OH	OCOCH ₃	OCOPh(4-Cl)	3.77	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
OCOPh(4-NO ₂)	OCOPh(4-NO ₂)	OH	4.31	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OCOPh	OCOPh	OCOPh	5.33	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OCOPh(4-Cl)	OCOPh(4-Cl)	OCOPh(4-Cl)	6.45	-	-	-	++	++	++	++	++	++	++	++	++	++	++	++	++	++
OH	O-FA	OH	>7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^aUnless otherwise stated, R₂ conformation is β; FA, fatty acid; log P values estimated using ChemBioDraw; -, not active (GI₅₀ > 30 μM); +, low cytotoxic (10 < GI₅₀ ≤ 30 μM); ++, cytotoxic (5 < GI₅₀ ≤ 10 μM); +++, highly cytotoxic (GI₅₀ ≤ 5 μM); Ph, phenyl group. ^bReferences in square brackets. ^cSee Figure 3 for chemical structure.

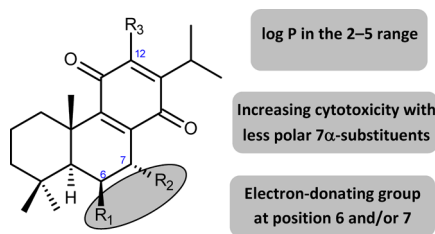


Figure 3. Proposed structure–activity relationships for 6,7,12-substituted royleanone-type abietane diterpenes based on data from Table 4.

human embryonal bronchial epithelial cells) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The multidrug-resistant non-small cell lung carcinoma cell line (NCI-H460/R) with P-glycoprotein overexpression was obtained from the original NCI-H460 cell line by continuous treatment with stepwise increasing concentrations of doxorubicin (5–100 nM) for 3 months according to a procedure optimized by Pesic et al.³⁴ Cell lines were routinely cultured in RPMI 1640 with ultraglutaramine or DMEM (MDA-MB-231 cells), supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and maintained under standard cell conditions at 37 °C under a humidified atmosphere containing 5% CO₂.^{50,51}

Plant Material. *Plectranthus madagascariensis* Benth. was cultivated in Parque Botânico da Tapada da Ajuda (Instituto Superior Agrário, Lisbon, Portugal) from cuttings obtained from the Kirstenbosch National Botanical Garden (Cape Town, South Africa), and voucher specimens (841/2007) were deposited in the Herbarium João de Carvalho e Vasconcellos (Instituto Superior Agrário, Lisbon, Portugal). The whole plant material was collected in 2007, dried at room temperature, and stored protected from light and humidity.

Extract Preparation. The dried plant was ground to small pieces, pulverized, and extracted with acetone or methanol by maceration (ME) or ultrasound-assisted extraction (UAE) and also by supercritical fluid extraction (SCFE) with supercritical CO₂ (scCO₂). Each crude extract was separated from the remaining plant material by paper filtration, and the organic solvents were evaporated in a rotary evaporator below 40 °C. Each dried extract was weighted and stored at –20 °C until further use.

The organic extracts were prepared by maceration (ME) or ultrasound-assisted (UAE) extraction by adding the plant material (10 g) to 200 mL of organic solvent (acetone or methanol) kept stirring for 24 h (ME) or sonicated in an ultrasonic bath at 35 kHz for 2 h (UAE). Alternatively, supercritical fluid extraction (SCFE) was also performed by packing the plant material (30 g) into a 278 cm³ inox cell and injecting scCO₂ during 3 h at a flow rate of 0.3 kg/h under a pressure of 230 bar and a temperature of 40 °C.⁵² The remaining plant material resulting from SCFE was recovered, air dried, and re-extracted (R-SCFE) by addition to 200 mL of acetone kept stirring for 24 h at room temperature.

Extraction and Purification of 7α-Formyloxy-6β-hydroxyroyleanone (3). Ultrasound-assisted extraction of *P. madagascariensis* by sonication of 100 g of plant material in 2 L of acetone for 2 h yielded 480 mg of dried residue (0.48% (w/w) extraction yield) after evaporation of the organic solvent. The extract was treated with activated charcoal to eliminate the high content in chlorophyll plant pigments and

fractionated by chromatography on a silica gel (30 g) column eluted with a gradient of ethyl acetate in hexane. The compound (3) was recovered from the fraction eluted with hexane/ethyl acetate 95:5 (v/v) and purified by preparative chromatography through repeated elution with hexane/ethyl acetate 75:25 (v/v) followed by recrystallization from methanol, yielding 6.1 mg (1.27% (w/w) yield) of yellow needles. Spectroscopic characterization was performed by one-dimensional (¹H and ¹³C NMR) and two-dimensional (COSY, HMBC, and HSQC) NMR (Table 2 and Figures S3 and S4, Supporting Information).

HPLC-DAD Fingerprinting. The extract profiling was performed with an Agilent Technologies 1200 Infinity Series LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a diode array detector (DAD) and processed using the ChemStation software. Four detection wavelengths were selected: 254, 270, 280, and 330 nm. A 20 μL sample was injected into a reverse phase LiChrospher 100 RP-18 5 μm (4.0 × 250 mm) column (Merck, Darmstadt, Germany) and eluted with a gradient composed of methanol (A), acetonitrile (B), and 0.3% (w/v) trichloroacetic acid in ultrapure water (C) as follows: 0 min, 15% A, 5% B, and 80% C; 20 min, 70% A, 30% B, and 0% C; 25 min, 70% A, 30% B, and 0% C; and 28 min, 15% A, 5% B, and 80% C. The flow rate was set at 1 mL/min at room temperature. Solvents were previously filtered and degassed through a 0.22 μm membrane filter. All analysis was performed in triplicate, and the results are presented as mean ± SD.

The major peaks from each extract sample were identified by comparing the retention time and UV–vis spectrum overlay with commercial standards (rosmarinic acid) or authentic standards previously obtained from *Plectranthus* spp.² The calibration curves were constructed as a linear regression of the analyte concentration (mM) against the average peak area. The limit of detection (LOD) and limit of quantification (LOQ) were determined to evaluate the sensitivity of the analysis corresponding to the concentrations of the analyte that resulted in signal-to-noise ratios of 3 (LOD) and 10 (LOQ) following the guidelines from ICH Q2(R1) on validation of analytical procedures.⁵³ The LOD and LOQ were calculated as LOD = 3.3σ/S and LOQ = 10σ/S, where σ corresponds to the standard deviation of the response and S corresponds to the slope of the calibration curve, which was estimated from the calibration curve of the analyte (Table S1, Supporting Information).

Cytotoxicity Assays. The cytotoxicity of compounds (1–5) obtained from *P. madagascariensis* extracts was evaluated in a battery of human cell lines (MDA-MB-231, MCF-7, and HCT116) using the sulforhodamine B (SRB) assay according to a procedure supervised by Saraiva et al.^{54,55} Briefly, cells were plated in 96-well plates (5.0 × 10³ cells/well) and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. Then, cells were exposed to serial dilutions of each compound (1.85–150 μM) and incubated for another 48 h. Following fixation with 10% trichloroacetic acid, cells were stained with 0.4% (w/v) sulforhodamine B and then washed with 1% acetic acid to remove the unbound stain. The adsorbed dye was dissolved with 10 mM Tris buffer (pH 10.5), and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA). Doxorubicin was used as positive control. In Table 3, Student's *t* test was used to compare the GI₅₀ values of compounds (3–5) to that of compound 2 in the MCF-7 cell line. All *P* values

were based on the two-sided statistical analysis, and $P < 0.05$ was considered to be statistically significant.

The cytotoxic effects of the compounds toward NCI-H460, NCI-H460/R, and MCR-5 cell lines were further assessed by the MTT colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a formazan dye by active mitochondria of living cells according to the procedure described by Pesic et al.^{34,56} Briefly, cells were plated in 96-well plates, inoculated with the compounds (2.5–50 μM), and incubated for 72 h at 37 °C in a humidified atmosphere with 5% CO_2 . Then, 100 μL of MTT solution (1 mg/mL) was added to each well, and the plates were incubated at 37 °C for 4 h. The formazan product was dissolved by adding 200 μL of DMSO per well, and absorbance was measured at 540 nm in a microplate reader (LKB 5060–006 Micro Plate Reader, Vienna, Austria). Paclitaxel was used as positive control.

Cell viability (%) was calculated as a fraction of the absorbance shown by nontreated cells (control), and the GI_{50} values, corresponding to the concentration of the compound that inhibits 50% of cell growth, were determined. At least two independent experiments in triplicate were performed for each test compound. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as mean \pm SD. Normality was checked by Column Statistics of GraphPad Prism 6 software—Shapiro–Wilk normality test. Accordingly, the unpaired t test from row data after the MTT measurement was applied.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00512.

¹H NMR and ¹³C NMR spectra of compound (3), HPLC-DAD analytical profile of plant extracts and UV-spectra overlay, and analytical parameters for HPLC-DAD quantification method (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: patricia.rijo@ulusofona.pt. Tel: 351 21 7515577. Fax: 351 21 7515598.

ORCID

Lucília Saraiva: 0000-0002-9531-4939

Milica Pesic: 0000-0002-9045-8239

Patrícia Rijo: 0000-0001-7992-8343

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Ernst van Jaarsveld (Kirstenbosch National Botanic Gardens, South Africa) for providing cuttings of the *Plectranthus* plants, Teresa Vasconcelos (Instituto Superior Agrário, Lisbon, Portugal) for the growing of the plants, and Maria João Cebola and Paula Pereira for the preparation of the supercritical fluid extracts. D.M. is thankful for grant PADDIC 2013-2014 (ALIES-COFAC), part of the PhD program in Health Sciences from Universidad de Alcalá and Universidade Lusófona. The authors would also like to thank to Fundação para a Ciência e Tecnologia for financial support under the

reference UID/DTP/04567/2016 and UID/MULTI/04378/2013.

■ REFERENCES

- (1) Lukhoba, C. W.; Simmonds, M. S. J.; Paton, A. J. *Plectranthus*: a review of ethnobotanical uses. *J. Ethnopharmacol.* **2006**, *103*, 1–24.
- (2) Gaspar-Marques, C.; Rijo, P.; Simões, M. F.; Duarte, M. A.; Rodríguez, B. Abietanes from *Plectranthus grandidentatus* and *P. hereroensis* against methicillin- and vancomycin-resistant bacteria. *Phytomedicine* **2006**, *13*, 267–271.
- (3) Rijo, P.; Faustino, C.; Simões, M. F. Antimicrobial natural products from *Plectranthus* plants. In *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*; Méndez-Vilas, A., Ed.; Formatex: Badajoz, 2013; pp 922–931.
- (4) Rijo, P.; Simões, M. F.; Francisco, A. P.; Rojas, R.; Gilman, R. H.; Vaisberg, A. J.; Rodríguez, B.; Moiteiro, C. Antimycobacterial metabolites from *Plectranthus*: royleanone derivatives against *Mycobacterium tuberculosis* strains. *Chem. Biodiversity* **2010**, *7*, 922–932.
- (5) Kubínová, R.; Pořízková, R.; Navrátilová, A.; Farsa, O.; Hanáková, Z.; Bačinská, A.; Cížek, A.; Valentová, M. Antimicrobial and enzyme inhibitory activities of the constituents of *Plectranthus madagascariensis* (Pers.) Benth. *J. Enzyme Inhib. Med. Chem.* **2014**, *6366*, 1–4.
- (6) Falé, P. L.; Borges, C.; Madeira, P. J. A.; Ascensão, L.; Araújo, M. E. M.; Florêncio, M. H.; Serralheiro, M. L. M. Rosmarinic acid, scutellarein 4'-methyl ether 7-O-glucuronide and (16S)-coleon E are the main compounds responsible for the antiacetylcholinesterase and antioxidant activity in herbal tea of *Plectranthus barbatus* ("falso boldo"). *Food Chem.* **2009**, *114*, 798–805.
- (7) Gurgel, A. P. A. D.; da Silva, J. G.; Grangeiro, A. R. S.; Oliveira, D. C.; Lima, C. M. P.; da Silva, A. C. P.; Oliveira, R. A. G.; Souza, I. A. *In vivo* study of the anti-inflammatory and antitumor activities of leaves from *Plectranthus amboinicus* (Lour.) Spreng (Lamiaceae). *J. Ethnopharmacol.* **2009**, *125*, 361–363.
- (8) Minker, C.; Sheridan, H.; O'Meara, J.; Johnse, L. V.; Hook, I.; Lobstein, A.; Frankish, N. *In vivo* and *in vitro* evaluation of anti-inflammatory activity and cytotoxicity of extracts of seven *Plectranthus* species. *Planta Med.* **2007**, *73*, No. 074.
- (9) Burmistrova, O.; Perdomo, J.; Simões, M. F.; Rijo, P.; Quintana, J.; Estévez, F. The abietane diterpenoid parvifloron D from *Plectranthus ecklonii* is a potent apoptotic inducer in human leukemia cells. *Phytomedicine* **2015**, *22*, 1009–1016.
- (10) Cerqueira, F.; Cordeiro-da-Silva, A.; Gaspar-Marques, C.; Simões, F.; Pinto, M. M. M.; Nascimento, M. S. J. Effect of abietane diterpenes from *Plectranthus grandidentatus* on T- and B-lymphocyte proliferation. *Bioorg. Med. Chem.* **2004**, *12*, 217–223.
- (11) Marques, C. G.; Pedro, M.; Simões, M. F. A.; Nascimento, M. S. J.; Pinto, M. M. M.; Rodríguez, B. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Med.* **2002**, *68*, 839–840.
- (12) Saeed, M. E. M.; Meyer, M.; Hussein, A.; Efferth, T. Cytotoxicity of South-African medicinal plants towards sensitive and multidrug-resistant cancer cells. *J. Ethnopharmacol.* **2016**, *186*, 209–223.
- (13) Hasibuan, P. A. Z.; Rosidah; Ilyas, S.; Nasution, M. P. Antioxidant and cytotoxic activities of *Plectranthus amboinicus* (Lour.) Spreng. extracts. *Int. J. Pharm. Teach. Pract.* **2013**, *4*, 755–758.
- (14) Ramalakshmi, P.; Subramanian, N.; Saravanan, R.; Mohanakrishnan, H.; Muthu, M. Anticancer effect of *Coleus amboinicus* (Karpporavalli) on human lung cancer cell line (A549). *Int. J. Dev. Res.* **2014**, *4*, 2442–2449.
- (15) Arcanjo, D. D. R.; Albuquerque, A. C. M.; Melo-Neto, B.; Santana, L. C. L. R.; Medeiros, M. G. F.; Citó, A. M. G. L. Bioactivity evaluation against *Artemia salina* Leach of medicinal plants used in Brazilian Northeastern folk medicine. *Braz. J. Biol.* **2012**, *72*, 505–509.

- (16) Borges, G. A.; Ferreira, J. F.; Elias, S. T.; Guerra, E. N. S.; Silveira, D.; Simeoni, L. A. Cytotoxic effect of *Plectranthus neochilus* extracts in head and neck carcinoma cell lines. *Afr. J. Pharm. Pharmacol.* **2016**, *10*, 157–163.
- (17) Agarwal, K. C.; Parks, R. E., Jr. Forskolin: a potential antitumorigenic agent. *Int. J. Cancer* **1983**, *32*, 801–804.
- (18) McEwan, D. G.; Brunton, V. G.; Baillie, G. S.; Leslie, N. R.; Houslay, M. D.; Frame, M. C. Chemoresistant KM12C colon cancer cells are addicted to low cyclic AMP levels in a phosphodiesterase 4-regulated compartment via effects on phosphoinositide 3-kinase. *Cancer Res.* **2007**, *67*, 5248–5257.
- (19) Burmistrova, O.; Simões, M. F.; Rijo, P.; Quintana, J.; Bermejo, J.; Estévez, F. Antiproliferative activity of abietane diterpenoids against human tumor cells. *J. Nat. Prod.* **2013**, *76*, 1413–1423.
- (20) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582.
- (21) Ladeiras, D.; Monteiro, C. M.; Pereira, F.; Reis, C. P.; Afonso, C. A. M.; Rijo, P. Reactivity of diterpenoid quinones: royleanones. *Curr. Pharm. Des.* **2016**, *22*, 1682–1714.
- (22) Yulianto, W.; Andarwulan, N.; Giriwono, P. E.; Pamungkas, J. HPLC-based metabolomics to identify cytotoxic compounds from *Plectranthus amboinicus* (Lour.) Spreng against human breast cancer MCF-7 cells. *J. Chromatogr. B* **2016**, *1039*, 28–34.
- (23) Wellson, J.; Grayer, R. J.; Veitch, N. C.; Kokubun, T.; Lelli, R.; Kite, G. C.; Simmonds, M. S. J. Insect-antifeedant and antibacterial activity of diterpenoids from species of *Plectranthus*. *Phytochemistry* **2006**, *67*, 1818–1825.
- (24) Matias, D.; Pereira, F.; Nicolai, M.; Roberto, A.; Saraiva, N.; Fernandes, A. S.; Simões, M. F.; Lanza, A. D.; Reis, C. P.; Rijo, P. Abietane diterpenes from *Plectranthus madagascariensis*: a cytotoxicity screening. *Planta Med.* **2014**, *80*, P11152.
- (25) Miyase, T.; Rüedi, P.; Eugster, C. H. Diterpenoide Drüsenfarbstoffe aus Labiaten: Coleone U, V, W und 14-O-Formyl-coleon-V sowie 2 Royleanone aus *Plectranthus myrianthus* BRIQ; cis-und-trans-A/B-6, 7-Dioxoroyleanon. *Helv. Chim. Acta* **1977**, *60*, 2770–2779.
- (26) Mei, S.-X.; Jiang, B.; Niu, X.-M.; Li, M.-L.; Yang, H.; Na, Z.; Lin, Z.-W.; Li, C.-M.; Sun, H.-D. Abietane diterpenoids from *Coleus xanthanthus*. *J. Nat. Prod.* **2002**, *65*, 633–637.
- (27) Coutinho, I.; Pereira, G.; Simões, M. F.; Côrte-Real, M.; Gonçalves, J.; Saraiva, L. Selective activation of protein kinase C- δ and - ϵ by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U). *Biochem. Pharmacol.* **2009**, *78*, 449–459.
- (28) Matias, D.; Bessa, C.; Simões, M. F.; Reis, C. P.; Saraiva, L.; Rijo, P. Natural products as lead protein kinase C modulators for cancer therapy. In *Studies in Natural Products Chemistry: Bioactive Natural Products*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 2016; pp 45–79.
- (29) Hensch, M.; Rüedi, P.; Eugster, C. H. Horminon, Taxochinon und weitere Royleanone aus 2 abessinischen *Plectranthus*-Spezies (*Labiateae*). *Helv. Chim. Acta* **1975**, *58*, 1921–1934.
- (30) Brits, G. J.; Selchau, J.; van Deuren, G. Indigenous *Plectranthus* (*Lamiaceae*) from South Africa as new flowering pot plants. In *XX International Eucarpia Symposium, Section Ornamentals, Strategies for New Ornamentals-Part I*; 2001, 165–170.
- (31) Van Zyl, R. L.; Khan, F.; Edwards, T. J.; Drewes, S. E. Antiplasmodial activities of some abietane diterpenes from the leaves of five *Plectranthus* species. *S. Afr. J. Sci.* **2008**, *104*, 62–64.
- (32) Abdel-Mogib, M.; Albar, H. A.; Batterjee, S. M. Chemistry of the genus *Plectranthus*. *Molecules* **2002**, *7*, 271–301.
- (33) Rijo, P.; Matias, D.; Fernandes, A. S.; Simões, M. F.; Nicolai, M.; Reis, C. P. Antimicrobial plant extracts encapsulated into polymeric beads for potential application on the skin. *Polymer* **2014**, *6*, 479–490.
- (34) Pesic, M.; Markovic, J. Z.; Jankovic, D.; Kanazir, S.; Markovic, I. D.; Rakic, L.; Ruzdijic, S. Induced resistance in the human non small cell lung carcinoma (NCI-H460) cell Line In Vitro by anticancer drugs. *J. Chemother.* **2006**, *18*, 66–73.
- (35) Cleator, S.; Heller, W.; Coombes, R. C. Triple-negative breast cancer: therapeutic options. *Lancet Oncol.* **2007**, *8*, 235–244.
- (36) Morse-Gaudio, M.; Connolly, J. M.; Rose, D. P. Protein kinase C and its isoforms in human breast cancer cells: relationship to the invasive phenotype. *Int. J. Oncol.* **1998**, *12*, 1349–1403.
- (37) Li, Z.; Wang, N.; Fang, J.; Huang, J.; Tian, F.; Li, C.; Xie, F. Role of PKC-ERK signaling in tamoxifen-induced apoptosis and tamoxifen resistance in human breast cancer cells. *Oncol. Rep.* **2012**, *27*, 1879–1886.
- (38) Lønne, G. K.; Masoumi, K. C.; Lennartsson, J.; Larsson, C. Protein kinase C δ supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway. *J. Biol. Chem.* **2009**, *284*, 33456–33465.
- (39) Yokoyama, G.; Fujii, T.; Tayama, K.; Yamana, H.; Kuwano, M.; Shirouzu, K. PKC δ and MAPK mediate G₁ arrest induced by PMA in SKBR-3 breast cancer cells. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 720–726.
- (40) Chaudhary, P. M.; Roninson, I. B. Activation of MDRI (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncol. Res.* **1992**, *4*, 281–290.
- (41) Al-Katib, A. M.; Smith, M. R.; Kamanda, W. S.; Pettit, G. R.; Hamdan, M.; Mohamed, A. N.; Chelladurai, B.; Mohammad, R. M. Bryostatins 1 down-regulates mdrl and potentiates vincristine cytotoxicity in diffuse large cell lymphoma xenografts. *Clin. Cancer Res.* **1998**, *4*, 1305–1314.
- (42) Fronza, M.; Murillo, R.; Ślusarczyk, S.; Adams, M.; Hamburger, M.; Heinzmann, B.; Laufer, S.; Merfort, I. *In vitro* cytotoxic activity of abietane diterpenes from *Peltodon longipes* as well as *Salvia miltiorrhiza* and *Salvia sahendica*. *Bioorg. Med. Chem.* **2011**, *19*, 4876–4881.
- (43) Fronza, M.; Lamy, E.; Günther, S.; Heinzmann, B.; Laufer, S.; Merfort, I. Abietane diterpenes induce cytotoxic effects in human pancreatic cancer cell line MIA PaCa-2 through different modes of action. *Phytochemistry* **2012**, *78*, 107–119.
- (44) Islam, M. T. Diterpenes and their derivatives as potential anticancer agents. *Phytother. Res.* **2017**, *31*, 691–712.
- (45) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
- (46) Areche, C.; Schmeda-Hirschmann, G.; Theoduloz, C.; Rodríguez, J. A. Gastroprotective effect and cytotoxicity of abietane diterpenes from the Chilean *Lamiaceae* *Sphacele chamaedryoides* (Balbis) Briq. *J. Pharm. Pharmacol.* **2009**, *61*, 1689–1697.
- (47) Kusumoto, N.; Aburai, N.; Ashitani, T.; Takahashi, K.; Kimura, K.-i. Pharmacological prospects of oxygenated abietane-type diterpenoids from *Taxodium distichum* cones. *Adv. Biol. Chem.* **2014**, *4*, 109–115.
- (48) Li, S.; Wang, P.; Deng, G.; Yuan, W.; Su, Z. Cytotoxic compounds from invasive giant salvinia (*Salvinia molesta*) against human tumor cells. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 6682–6687.
- (49) Jonathan, L. T.; Che, C.-T.; Pezzuto, J. M.; Fong, H. H. S.; Farnsworth, N. R. 7-O-Methylhorminone and other cytotoxic diterpene quinones from *Lepechinia bullata*. *J. Nat. Prod.* **1989**, *52*, 571–575.
- (50) Fernandes, A. S.; Serejo, J.; Gaspar, J.; Cabral, F.; Bettencourt, A. F.; Rueff, J.; Castro, M.; Costa, J.; Oliveira, N. G. Oxidative injury in V79 Chinese hamster cells: protective role of the superoxide dismutase mimetic MnTM-4-PyP. *Cell Biol. Toxicol.* **2010**, *26*, 91–101.
- (51) Guerreiro, P. S.; Fernandes, A. S.; Costa, J. G.; Castro, M.; Miranda, J. P.; Oliveira, N. G. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. *Mutat. Res.* **2013**, *757*, 140–147.
- (52) Pereira, P.; Bernardo-Gil, M. G.; Cebola, M. J.; Mauricio, E.; Romano, A. Supercritical fluid extracts with antioxidant and antimicrobial activities from myrtle (*Myrtus communis* L.) leaves. Response surface optimization. *J. Supercrit. Fluids* **2013**, *83*, 57–64.
- (53) ICH Harmonised Tripartite Guideline Q2(R1). Validation of Analytical Procedures: Text and Methodology. In *International*

Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; European Medicines Agency: Chicago, IL, 2005.

(54) Leão, M.; Soares, J.; Gomes, S.; Raimundo, L.; Ramos, H.; Bessa, C.; Queiroz, G.; Domingos, S.; Pinto, M.; Inga, A.; Cidade, H.; Saraiva, L. Enhanced cytotoxicity of prenylated chalcone against tumour cells via disruption of the p53-MDM2 interaction. *Life Sci.* **2015**, *142*, 60–65.

(55) Soares, J.; Pereira, N. A. L.; Monteiro, Â.; Leão, M.; Bessa, C.; dos Santos, D. J. V. A.; Raimundo, L.; Queiroz, G.; Bisio, A.; Inga, A.; Pereira, C.; Santos, M. M. M.; Saraiva, L. Oxazoloisindolinones with *in vitro* antitumor activity selectively activate a p53-pathway through potential inhibition of the p53-MDM2 interaction. *Eur. J. Pharm. Sci.* **2015**, *66*, 138–147.

(56) Fishedick, J. T.; Pesic, M.; Podolski-Renic, A.; Bankovic, J.; de Vos, R. C. H.; Perić, M.; Todorović, S.; Tanic, N. Cytotoxic activity of sesquiterpene lactones from *Inula britannica* on human cancer cell lines. *Phytochem. Lett.* **2013**, *6*, 246–252.