A C_{20}-*nor*-abietane and three abietane-type diterpenoids from *Plectranthus mutabilis* Codd. leaves as modulators of P-glycoprotein activity

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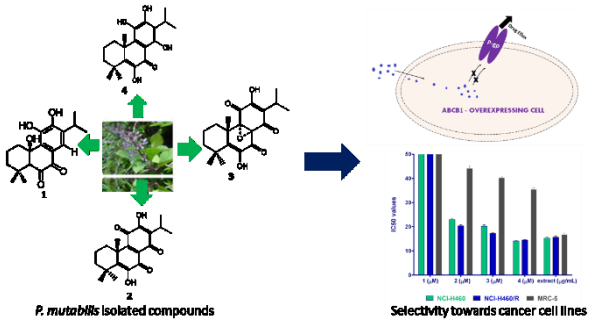


ABSTRACT: In this study, a bioguided fractionation of *P. mutabilis* extract was performed by chromatographic methods. It yielded one new nor-abietane diterpene, mutabilol (1), and three known abietanes, coleon-U-quinone (2), 8α,9α-epoxycoleon-U-quinone (3), and coleon U (4). An abietane diterpenoid (5) was also tentatively identified using HPLC-MS/MS. Moreover, the extract profile and quantification of each isolated compound were determined by HPLC-DAD. Compound 4 was the major compound in the extract. Compounds 2 - 4 were found to be selective towards the cancer cell lines and were able to inhibit P-gp activity in NCI-H460/R cells at longer exposure of 72 h and consequently revert doxorubicin (DOX) resistance in subsequent combined treatment. None of the compounds influenced the P-gp expression in NCI-H460/R cells, while the extract significantly increased it.

P-gp is one of the major contributors to multidrug resistance (MDR) as it protects MDR cancer cells by effluxing cytotoxic drugs [1]. It extrudes a wide range of therapeutic drugs from cancer cells including paclitaxel, doxorubicin, daunorubicin, epirubicin, mitoxantrone, vincristine, and vinblastine [2], decreasing their intracellular concentration and rendering their efficacy [3]. In this context, natural products are considered as an alternative source of drugs to fight cancer MDR [4]. Phytochemical studies on *Plectranthus* (Lamiaceae) genus revealed the presence of bioactive abietane-type diterpenoids [5–9] capable of inhibiting P-gp activity [10,11].

P. mutabilis Codd. is a succulent herb grown mainly in South Africa [12]. The preliminary screening study, *P. mutabilis* extract was found to have cytotoxic activity in different cancer cell lines [13]. It is, therefore, necessary to enrich the phytochemical information of this plant in literature and identify the compounds that may be responsible for its cytotoxicity. In this work, the isolated compounds and the acetone extract were tested against sensitive and MDR non-small cell lung carcinoma cells as well as normal embryonic lung fibroblasts to assess: i) their selectivity towards cancer cells, ii) their effects against MDR phenotype, iii) the interaction with P-gp, and iv) the potential to sensitize MDR cancer cells to doxorubicin (DOX).

The air-dried powdered *P. mutabilis* Codd. leaves (SI plant material) (0.71 kg) were extracted with acetone (5 x 8.75 L each 1h) at room temperature using the ultrasound-assisted extraction method (ultrasound apparatus from VWR). The solvent was evaporated under reduced pressure at 40 °C and 44.07 g of crude extract obtained. This extract was subjected to flash column chromatography over silica gel (Merck 9385, 200 g) using mixtures of *n*-hexane: EtOAc (1:0 to 0:1) and EtOAc: MeOH (1:0 to 0:1) as eluents. According to differences in composition as indicated by TLC, ten crude fractions were obtained (A1 to A10).

A bioguided isolation was done by evaluating the general toxicity of the crude fractions using the *Artemia salina* model [20]. The most bioactive fraction (A7), eluted with *n*-hexane: EtOAc (80:20), was fractionated by column chromatography over silica gel (100g) using *n*-hexane: EtOAc (9:1) as eluent to give 9 fractions (B_1-1 to B_1-9). The fractions B_1-5 were subjected to column chromatography (silica gel, 152g) using *n*-hexane: CH_2Cl_2 (3:2) as eluent to obtain five fractions (C_1-1 to C_1-5). Fraction C_1-4 was purified employing a Combiflash instrument on polyamide stationary phase (100 g) using gradient elution with CH_2Cl_2: MeOH (1: 0 to 3: 22) at a flow rate of 60 mL/min that afforded compound **2** (3.7 mg; 0.0084 % m/m).

Subsequently, fraction C_1-5 (divided into two aliquots) was successively rechromatographed using the Combiflash system with polyamide columns (120 g and 25g), and with flow rates of 60 mL/min and 30 mL/min respectively in gradient elution mode with CH_2Cl_2: MeOH (1: 0 to 9:1) as eluents, to afford compound **4** (152.1 mg; 0.3451% m/m).

Fraction E_2-1 was further separated by means of preparative reverse- phase high performance liquid chromatography (RP-HPLC) using 53% aqueous acetonitrile as mobile phase, to afford compound **3** (63 mg; 0.143 % m/m). Fraction C_1-3 was further purified by flash column chromatography (silica gel, 24g; 25 mL/min) on the Combiflash instrument with *n*-hexane: EtOAc (87.5:12.5) and subsequently, by reverse-phase flash chromatography (C18; 30 g; 35 mL/min) with MeOH: H_2O (55% to 100% MeOH) to afford compound **1** (10.2 mg; 0.023 % m/m).

This *P. mutabilis* leaves acetone extract was subject to a bioguided chromatographic fractionation based on its toxicity on the *Artemia salina* model [13](supplementary information, SI). A new C_{-20} *nor*-abietane, named mutabilol [(+)-5*S*,10*R*-10,11,12-trihydroxy-6,7-dioxo-20-*nor*abieta-8,11,13-triene, **1**) was isolated, together with three known abietane diterpenes, coleon-U-quinone (**2**), 8α,9α-epoxycoleon-U-quinone (**3**) and coleon U (**4**) (Figure **1**). Their structures were elucidated by spectroscopic methods. The spectroscopic data of compounds **2-4** were in agreement with those reported in the literature [14–16] (S**2**).

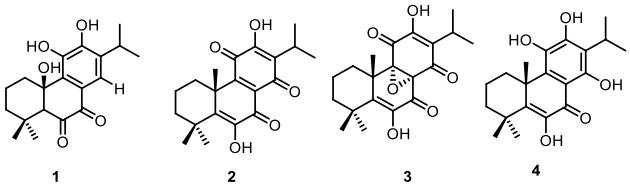


Figure 1. Structures of compounds 1–4 from *P. mutabilis*

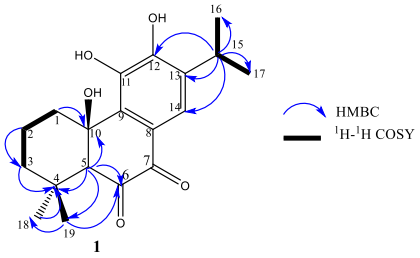


Figure 2. Key 1H–1H COSY (bold lines) and HMBC (blue arrows) correlations compound 1

Compound **1**, named mutabilol, was isolated as a purple solid. Its molecular formula was determined as $C_{19}H_{24}O_5$ from the HRMS that showed a protonated molecular ion at 333.1694 indicative of 8 degrees of unsaturation. In the IR spectrum, characteristic absorption bands for a hydroxyl group (3388.3 cm^{-1}) and carbonyl groups (1779.3), including a conjugated one (1656.3 cm^{-1}) were observed. The ^{1}H-NMR spectrum (Table S**1**) indicated the presence of an isopropyl substituent evidenced from the characteristic downfield signal at δ_H 3.16 (1H, sept, J = 7.1 Hz, H-15) and two doublet methyl groups at δ_H 1.18 (3H, d, J = 6.9 Hz, CH_3-16), and 1.17 (3H, d, J = 6.9 Hz, CH_3-17). Moreover, ^{1}H NMR spectrum also showed the signals for one aromatic proton at δ_H 7.23 (H-14), a tertiary methine signal at δ_H 1.26 (H-5), two singlet methyl groups at δ_H 1.29 and 0.94 (H-18 and H-19), and three hydroxyl groups at δ_H 8.05 (12-OH), 7.76 (10-OH), and 7.56 (11-OH). The ^{13}C-NMR, HMBC, and HSQC (supplementary information) displayed resonances of nineteen carbons corresponding to four methyl groups, three methylenes, three methines (one sp^2 carbon at 115.9), and nine quaternary carbons (including one oxygenated at 54.2 and two carbonyl groups at 176.4 and 178.9).

The ^{1}H- and ^{13}C-NMR spectra of compound **1** were similar to those of coleon V (Supplementary information) [15]. The main differences were the absence of the C-20 methyl group signal (δc 23.6) that together with a downfield quaternary carbon at δc 54.2 suggested the presence of an extra hydroxyl group at C-10 and the nor-abietane structure. Moreover, the absence of a hydroxyl group at C-14 was indicated by the signal at δ_H 7.23 instead of δ_H 13.47, and the downfield shift of C-14 (δc 115.9).

These structural features were confirmed by 2D NMR data analysis (COSY, HMQC, and HMBC (Figure **2**). In particular, the $^2J_{C\text{-}H}$ correlations observed between the quaternary carbon at δ_c 54.2 and H1-β (δ_H 2.85) and H-5 (δ_H 1.26) corroborated the presence of the

hydroxyl group at C-10. The cross-peak between the carbonyl signal at δ_c 176.4 and H-5 was used to assign its position at C-6. Furthermore, the long-range correlation of C-14 (115.96) to H-15 (δ_H 3.16) confirmed the absence of a hydroxyl group at C-14. Based on the above evidence, the structure of compound **1** was identified as (+)-(5*S*,10*R*)-10,11,12-trihydroxy-6,7-dioxo-20-*nor*-abieta-8,11,13-triene.

LC-MS/MS was used to study the fragmentation pattern of the isolated compounds and further analyse the presence of diterpenoids in the acetone extract. The UHPLC-MS/MS experiments were carried out on a Waters AcquityTM Ultra High Performance LC (Waters®, Ireland) system equipped with a binary pump, solvent degasser, auto sampler, and column oven. Chromatographic separation was achieved on a Purospher® STAR RP-18 2 µm (2.1 x 50 mm) column Compound **1** yielded its deprotonated ion at m/z 331 $[M-H]^-$. Its MS/MS spectra showed fragments at 303 due to the loss of -CO group $[M-H-CO]^-$ and m/z 313 resulting from the loss of water $[M-H-H_2O]^-$. Other product ions at m/z 298 $[M-H-H_2O-CH_3]^-$, 285 $[M-H-CO-H_2O]^-$ and 259 $[M-2H-CO-C_3H_7]^-$ were also detected at low abundance. Compound **(2)** showed a protonated ion at m/z 345 $M+H]^+$ that generated fragments m/z 111 and m/z 233 resulting from the fragmentation at the 11,13-positions of the C-ring [22]. Fragment m/z 303 corresponds to the loss of the propene unit $[M+H-43Da]^+$ and 327 from the loss of H_2O $[M+H-H_2O]^+$. The fragmentation of the parent ion of compound **3** (m/z 359 $[M-H]^-$ produced an ion at m/z 300 $[M-H-C_3H_7O]^-$ and a base peak at m/z 285 $[M-H-2CO-H_2O]^-$[23]. The fragment ion at m/z 272 $[M-H-C_3H_7O-CO]^-$ was also observed. Compound **4** showed a deprotonated ion at m/z 345 $[M-H]^-$ and a base peak at m/z 330 due to the loss of a methyl group.

The UHPLC-MS/MS analysis revealed an additional compound in the extract. This compound exhibited a protonated ion at 389 $[M+H]^+$. Its MS/MS spectrum showed an abundant fragment ion at m/z 347 that was attributed to the loss of a propene unit $[M+H-C_3H_6]^+$, suggesting the presence of the characteristic isopropyl group of abietane diterpenoids. The presence of a small fragment ion at m/z 330 $[M+H-C_2H_3O_2]^+$ suggested the loss of an acetoxy group (59 Da) (Supplementary information). The above finding suggests that this compound could be an acetoxy derivative of an abietane diterpenoid. More work is ongoing for the proper identification of this compound.

HPLC analysis of the *P. mutabilis* extract revealed the presence of the following compounds mutabilol tR = 9.20 (**1**), Coleon U quinone tR = 10.22 (**2**), 8α,9α-Epoxycoleon U quinone, tR = 9.43 (**3**), Coleon U, tR = 11.80 (**4**). Compound **4** was found to be the major compound in the extract (Table **2**). Coleon U quinone appears to be an oxidation product of coleon U, as it was only present in the crude extract in small quantities. These were in agreement with the results obtained by Diogo *et al*, from *P. madagascariensis* Benth. [7] and Wellsow et al., 2006, where Coleon U quinone only appeared in significant amounts during the isolation process [16].

The biosynthetic relationship between compounds **2-4** was therefore considered employing a computational study. While Coleon U **4** is the undoubted precursor of **2** and **3**, the formation of epoxyquinone **3** can be formulated straight from Coleon U **4** or from its quinone **2**. To get insight into the formation of epoxyquinone **3**, the electron density-based local reactivity descriptors condensed Fukui indexes have been determined for the three compounds, together with the bond dissociations energies (BDEs) of the O-H bonds of Coleon U **4 (**Table **3)**. Figure **3** depicts the results of the electrophilic (f_k^-), nucleophilic (f_k^+), and radical (f_k^0) Fukui functions in the gas phase, where the positions with higher Fukui indexes have been indicated. In table **1,** is presented the bond dissociation energies of the O-H bonds present in Coleon U **4**. The BDE of the O-H at the C11 position is significantly lower than any of the other O-H bonds, which indicates that under oxidative radical conditions the removal of this hydrogen radical will trigger the oxidative process of the hydroquinone to quinone **2**. The analysis of the Fukui reactivity descriptors of **2** and **4** point to the carbon atoms of the hydroquinone moiety as the most nucleophilic region of the molecule while the quinone portion becomes the most electrophilic region in **2**. This observation suggests that the installation of epoxide in **2** to form **3** would occur via the nucleophilic conjugate addition by an oxygen-carrier group (eg. hydroperoxide). On the other hand, the oxygen atom in the epoxide of **3** is more likely to have its origin in electrophilic molecular oxygen making Coleon U **4** a suitable biosynthetic precursor considering its nucleophilic character around the hydroquinone core. With this in mind, the formation of anions $\mathbf{4^{11}}$ and $\mathbf{4^{14}}$ was considered, and the electrophilic Fukui functions (f_k^-) of both anions were analyzed (Figure **4**). The difference of 25 kcal/mol in the Gibbs free energy of formation of both anions indicates that the hydroxyl deprotonation at position 11 is preferable. The analysis of the electrophilic Fukui function (f_k^-) indicates position C9 as the second most nucleophilic atom (0.136 condensed index), only overpassed by the anionic oxygen. A similar analysis for the electrophilic Fukui function (f_k^-) for anion $\mathbf{4^{14}}$ indicates C11 as the most nucleophilic carbon, although having a close condensed index to C9 and C13 (0.138 *vs* 0.122-0.124).

Table 2. Abietane Diterpenoids compositions of the extract from P. mutabilis by HPLC–DAD

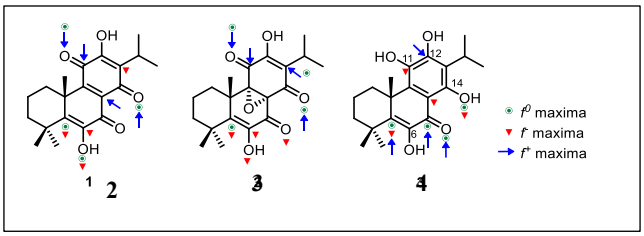
Compounds	Concentration µg/mg	LOD µg/mg	LOQ µg/mg
Mutabilol (1)	51±0.008	1.120	3.39
Coleon U quinone (2)	35±0.005	0.102	0.310
8α,9α-Epoxycoleon U quinone (3)	36±0.018	0.828	2.510
Coleon U (4)	96±0.048	0.78	2.35

With this in mind, two paths are suggested as plausible for the formation of epoxide **3** from **4** (Scheme 1). Upon hydroxyl deprotonation of **4** at the C11 position, the anion $\mathbf{4^{11}}$ reacts with molecular oxygen at C9 to form a hydroperoxide, which undergoes nucleophilic attack at C11 carbonyl to deliver a dioxetane. A proton exchange places the anion at the oxygen at C14 position, followed by dioxetane opening delivers the epoxide. Alternatively, the same dioxetane can be reached from anion $\mathbf{4^{14}}$ through the electrophilic attack at molecular oxygen by C11, as corroborated by the higher nucleophilic Fukui condensed index, followed by conjugate addition. While the formation of $\mathbf{4^{11}}$ is more favorable than the formation of $\mathbf{4^{14}}$, given its lower Gibbs free energy of formation, the oxygen electrophilic addition to C9 formulated in the first path should be hampered by stereochemical constraints. Furthermore, the formation of epoxyquinones [17,18] through electrophilic attack by phenol bearing carbons (such as in C11) has been reported in bacteria [19], fungi [20], and in mammals in the biosynthesis of vitamin K epoxide [21,22].

Table 1. NMR data of compound 1 (CD3OD, 1H 500 MHz, 13C 126 MHz; δ in ppm, J in Hz).

Position	δ_H	δ_C
1α	1.78 (m)	
1β	2.84 (m)	35.8
2α	1.56 (m, J = 3.4)	
2β	1.70 (m, J = 14.0, 3.4 Hz)	18.7
3α	2.89 (m)	
3β	1.64 (m)	32.9
4		34.7
5	1.26 (s)	42.4
6		176.4
7		178.9
8		125.3
9		153.2
10-OH	7.76 (s)	54.2
11-OH	7.56 (s)	170.6
12-OH	8.05 (s)	175.5
13		118.8
14	7.23 (s)	115.96
15	3.16 (sept, J = 7.1)	24.8
16	1.18 (d, J = 6.9)	21.5
17	1.17 (d, J = 6.9)	19.5
18	0.94 (d, J = 4.9)	28.2
19	1.29 (d, J = 4.9)	30.3

Figure 3. Electrophilic (f_k^-), nucleophilic (f_k^+) and radical (f_k^0) Fukui functions of **2-4**. The higher condensed Fukui indexes are indicated as green circles, red triangles, and blue arrows, respectively representing the sites in the molecules that are most susceptible to a radical attack, most nucleophilic, and most electrophilic.



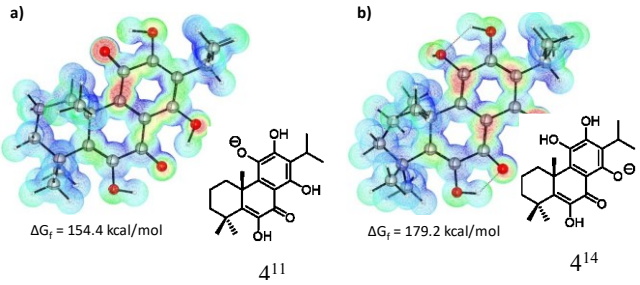


Figure 4. Electrophilic Fukui functions (f_k^-) and Gibbs energy for the formation of anions derived from deprotonation of Coleon U (**4**) at hydroxyl positions on C11 a) and C14 b).

The viability of human cancer cells was compared with that of human normal cells upon treatment with the acetone extract (2, 5, 10, 20, and 50 μg/mL) and diterpenes **1**-**4** (2, 5, 10, 20, and 50 μM), isolated from *P. mutabilis*. The largest reduction of cancer cell viability (metabolic activity) was observed with coleon U - compound **4** (IC_{50} ≈ 14 μM) against the NCI-H460 and NCI-H460/R expressed with P-gp which also showed the highest selectivity index at 2.5 (Table S**5**).

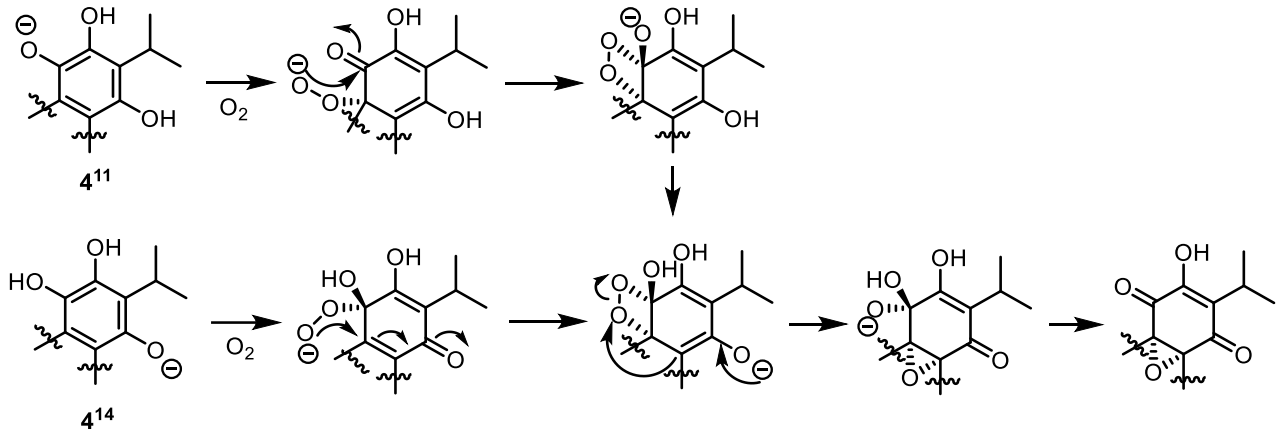
Compounds **2** and **3** (coleon U quinone and 8α,9α-epoxycoleon U quinone, respectively) showed similar inhibition of cancer cell viability (IC_{50} ≈ 20 μM) and a similar selectivity index of 2.0. New identified compound **1** - mutabilol was inactive against all three cell lines displaying IC_{50} over the highest concentration for compounds used in the study. The acetone extract showed similar activity against cancer and normal cells with a selectivity index of 1.0. Importantly, the presence of MDR phenotype (P-gp overexpression) in NCI-H460/R did not affect the inhibitory effect of **2**, **3**, **4,** and the extract, implying that they are not substrates for P-gp.

All tested compounds, as well as extract, decreased the Rho123 accumulation in NCI-H460/R cells. This finding implies that compounds and extract stimulate P-gp activity in a direct interaction assessed after 30 min (Table S**6**).

Based on these results, we studied Rho123 accumulation after 72 h treatment with compounds and extract. All compounds except 4 and extract increased Rho123 accumulation when 10 μM was administrated (Table S**7**). However, 10 μM of **4** decreased the accumulation of Rho123 as well as 10 μg/mL of extract.

Table 3. Hydrogen Bond Dissociation Energies (BDEs; kcal/mol) of O-H bonds in 4.

Position of OH in 4	O-H Bond Dissociation Energy (kcal/mol)
C6	103.8
C11	84.1
C12	103.0
C14	116.5



Scheme 1

The effect on P-gp (ABCB1) expression was assessed by flow cytometry using FITC conjugated ABCB1 antibody after 72 h treatment of NCI-H460/R cells with compounds and extract. NCI-H460 cells with almost null expression of ABCB1 were used as a negative control, while treatment with TQ in NCI-H460/R cells was used as a positive control. The results showed that none of the tested compounds had an influence on ABCB1 expression in NCI-H460/R cells, while the extract significantly increased ABCB1 expression in a concentration-dependent manner (Figure 5). The increase in ABCB1 expression induced by 10 µg/mL of extract was like the one achieved by 50 nM of TQ (Figure **5**). According to our results, the extract can increase resistance by stimulating P-gp expression. Therefore, it would not be wise to use the extract in combination with chemotherapeutics which are P-gp substrates.

Our findings prompted us to investigate the sensitizing effect of **2**, **3**, and **4** in NCI-H460/R cells. These three compounds showed an anticancer effect in MTT assay; a considerable increase of Rho123 accumulation after 72 h when applied in 5 µM (Table S**5**); and no influence on the ABCB1 expression in NCI-H460/R cells (Figure 5). Therefore, we studied the sensitization of NCI-H460/R cells to DOX which was combined with **2**, **3,** and **4.** DOX (0.1, 0.2, 0.5, 1 and 2 µM) was administrated after 72 h pre-treatment with **2**, **3,** and **4** applied in three concentrations below their IC_{50} (1, 2, and 5 µM). All subsequent combinations of all compounds with DOX showed significant reversal potential expressed as Relative reversal index (Table **4**). Relative reversal index was calculated as the ratio between IC_{50} value for DOX alone and IC_{50} value for DOX in combination with a compound. The most potent sensitization of NCI-H460/R cells to DOX was achieved with 5 µM of 2 and 4 with relative reversal index 7.465 and 6.045, respectively (Annex 6, Supplementary information. Fig. SI-5).

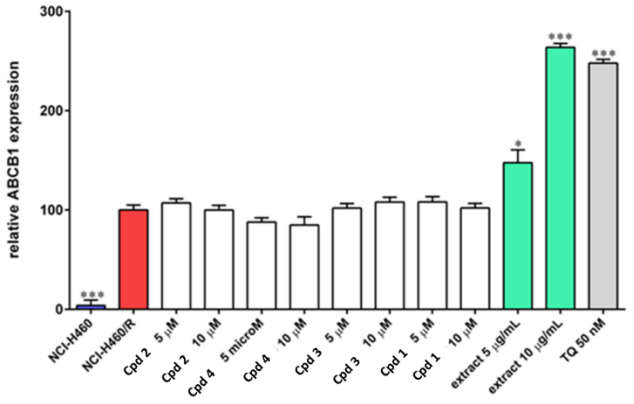


Figure 5. Effects of compounds and extract on the ABCB1 expression. Mean fluorescence of untreated NCI-H460/R cells was set to 100 and used for the comparison of other mean fluorescence (untreated NCI-H460 cells and treated NCI-H460/R cells) in GraphPad Prism 6 software (unpaired t-test). Mean fluorescence was calculated from three independent experiments. Significant difference to untreated NCI-H460/R cells was considered if $p < 0.05$ (*), $p < 0.001$ (***).

Table **4**. Reversal of DOX resistance in NCI-H460/R cells pre-treated with 1, 2, and 3, assayed by MTT

Compounds	Concentration	IC_{50} for DOX	Relative reversal index
DOX		1.620±0.084	
2	1 μM	0.565±0.012	2.867***
	2 μM	0.482±0.010	3.361***
	5 μM	0.217±0.004	7.465***
3	1 μM	0.820±0.016	1.976***
	2 μM	0.345±0.007	4.696***
	5 μM	0.343±0.006	4.723***
4	1 μM	0.625±0.013	2.592***
	2 μM	0.540±0.012	3.000***
	5 μM	0.268±0.006	6.045***

ASSOCIATED CONTENT

Supporting Information

Structure elucidation of compounds and NMR Data **of compounds 2**, **3** and **4**. Bio-guided screening of *P. mutabilis* column fractions for general toxicity at a concentration of 10 ppm using the *Artemia salina* test. Calibration curves for the HPLC quantification of diterpenoids from the acetonic extract of *P. Mutabilis*, ^{1}H NMR, COSY, HMBC, HSQC, ^{13}C–NMR spectra for new compounds. LCMS data for extract and isolated compounds, Reversal of DOX resistance in NCI-H460/R cells in subsequent treatment with compounds 2, 3, and 4, and atomic coordinates for all the optimized species (PBE1PBE/6-31G**). This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through the contributions of all authors. / All authors have approved the final version of the manuscript. / ‡These authors contributed equally.

Conflicts of Interest: "The authors declare no conflict of interest."

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