

Screening of Some Saponins and Phenolic Components of *Tribulus terrestris* and *Smilax excelsa* as MDR Modulators

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Abstract. Background: Cytotoxic activity of saponins and phenolic compounds have been described in the literature, but no reports were found on their multidrug resistance (MDR)-modulating effects on human *mdr1* gene-transfected mouse lymphoma cell line. Materials and Methods: Methylprototribestin, structurally related compounds and a mixture of 3 acetylated isomers of methylprotodioscin were investigated for antiproliferative effect and modulation of drug accumulation. Results: The growth inhibitory dose (ID₅₀) of the compounds ranged from 12.64 to 20.62 µg/ml. Methylprototribestin was the most effective resistance modifier. However, methylprotodioscin, pseudoprotodioscin, prosapogenin A of dioscin, tribestin and 5-*O*-caffeoylshikimic acid showed moderate MDR reversal activity. In a checkerboard method, methylprototribestin and the mixture of the 3 acetylated isomers enhanced the antiproliferative effects on MDR cells in combination with doxorubicin. Conclusion: Based on these results, methylprototribestin and the mixture of the 3 acetylated isomers can be recommended for further in vivo experiments in combination with anthracyclines in human MDR-cancer xenograft transplanted mice.

Multidrug resistance (MDR) is associated with overexpression of the membrane protein P-glycoprotein (P-gp; 170 kDa) (1), which expels anticancer drugs from cells and thereby leads to the ineffectiveness of chemotherapy. To overcome this problem, various individual compounds of synthetic and natural origin have been screened for their ability to reverse MDR (2-4).

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Efforts are continuing in the search for lead compounds for the development of products effective in chemotherapy.

We have directed our attention to steroid saponins, which are widely distributed in the plant kingdom. They possess a range of biological activities including cytotoxicity against some cell lines from leukaemia and solid tumours (5-8). The ability of steroid saponins to act as MDR modulators has not been explored to date.

The medical plants *Tribulus terrestris* (Zygophyllaceae) and *Smilax excelsa* (Liliaceae) growing in Bulgaria are among the richest producers of saponins. *Smilax* species have long been used in traditional medicine in many countries for the treatment of infectious diseases, skin disorders and liver inflammation (9, 10). *T. terrestris* is mainly known for its effectiveness in libido disorders, impotence and infertility. Data have also been published on its cardiovascular, cytotoxic and antimicrobial activities (11). The steroidal saponins have been considered to be the main active principles of these plants, responsible for their numerous healing applications.

In the present study, 6 pure steroidal saponins, a mixture of 3 saponin isomers, and 2 phenolics were isolated from the saponin fractions of *T. terrestris* and *S. excelsa*. All these compounds were evaluated as MDR modulators on multidrug-resistant mouse lymphoma cells.

Materials and Methods

Tested compounds. The air-dried milled rhizomes of *Smilax excelsa* (2.0 kg) were extracted three times with MeOH (3×7l×24 h) at room temperature, which led after concentration to 213.2 mg of material in the extract, which was resuspended in a mixture of MeOH/H₂O. This suspension was successively extracted with CHCl₃ (3×500 ml) and *n*-BuOH (3×500 ml) to give the corresponding CHCl₃ (6.0 g) and *n*-BuOH (27.5 g) extracts. From the *n*-BuOH extract, we succeeded in isolating methylprotodioscin (1); pseudoprotodioscin (3); dioscin (8); a mixture of 3 saponin isomers: (3-*O*-[2-*O*- (5), (3-*O*-[3-*O*- (6) and (3-*O*-[4-*O* (7) -acetyl- α -L-rhamnopyranosyl-(1→2)- α -L-rhamnopyranosyl-(1→4)]- β -D-glucopyranosyl]-26-*O*-[β -D-glucopyranosyl]-22 α -methoxy-(25R)-furost-5-ene-3 β ,26-diols; 5-*O*-

caffeoylshikimic acid (**10**); and *trans*-resveratrol (**11**) were isolated (Figures 1 and 2) (12, 13).

Air-dried and powdered plant material (5.5 kg) of *Tribulus terrestris* was extracted with 70% EtOH (3×6l×24 h) at room temperature. The combined EtOH solutions were concentrated to a small volume and extracted in succession with CHCl₃ (3×11×24 h) and *n*-BuOH (3×11×24 h). The *n*-BuOH layer was concentrated to dryness, giving the crude saponin extract (100.0 g). Liquid vacuum chromatography of this crude saponin extract (10.0 g) over silica gel (20.0 g) using CHCl₃/MeOH/H₂O (6:1:0.1 to 1:1:0.1) yielded fractions F1–F7. From fraction F4 (0.7 g), methylprotodioscin (**1**), methylprototribestin (**2**) and pseudoprotodioscin (**3**) were obtained (Figure 1) (14, 15). Fraction F2 (0.52 g) was subjected to medium-pressure liquid chromatography using gradient elution with MeOH/H₂O (4:6 to 8:1); 15 fractions were collected. The fractions eluted with MeOH/H₂O 7:3 and 4:1 were combined as subfractions R1 (0.11 g) and R2 (0.21 g). On silica gel column chromatography with CHCl₃/MeOH/H₂O (6:1:0.1 to 4:1:0.1), R2 gave dioscin (**8**, 30 mg) and tribestin (**9**, 16 mg) (16). From R1 (0.11 g) by preparative thin-layer chromatography (CHCl₃/MeOH/H₂O, 6:1:0.1), prosapogenin A of dioscin (4, 9 mg) was obtained. Prosapogenin A of dioscin was identified on the basis of its mass, 1D and 2D NMR spectra by comparison with literature data (17-19). This identification was confirmed by direct comparison (co-thin-layer chromatography) with an authentic sample obtained by solvolysis of tribestin (**9**) (Figure 1) (16).

Cell cultures. L5178 mouse T-cell lymphoma cells (U.S. FDA, USA) were transfected with pHa MDR1/A retrovirus (20). The *mdr1*-expressing cell line was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178 (parental) mouse T-cell lymphoma cells and the human *mdr1*-transfected subline were cultured at 37°C in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The mouse lymphoma cell line was maintained in a 5% CO₂ atmosphere.

Assay for reversal of MDR in tumour cells. The cells were adjusted to a density of 2×10⁶ cell/ml, resuspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. The tested compounds were added at different final concentrations (4.0 and 40 µg/ml), and the samples were incubated for 10 min at room temperature. Ten µl (5.2 µM final concentration) of the indicator rhodamine 123 (Sigma, St Louis, MO, USA) was added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured with a FACS Star Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil (EGIS Pharmaceuticals PLC, Budapest, Hungary) was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. An activity ratio (R) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$R = \frac{MDR\ treated / MDR\ control}{parental\ treated / parental\ control}$$

Assay for antiproliferative effect. The effects of increasing concentrations of the drugs alone and their combinations with resistance modifiers on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in two-steps in a volume of 50 µl to a final concentration of 25 µg/ml. A total of 1×10⁴ cells in 0.1 ml of medium were then added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C for 72 h, at the end of which 20 µl of MTT solution (thiazolyl blue solved in PBS to a final concentration of 5 mg/ml) were added to each well. After further incubation at 37°C for 4 h, 100 µl of sodium dodecyl sulphate (SDS) solution (10%) were measured into each well and the plates were further incubated at 37°C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of cell growth was determined as a percentage according to the formula:

$$100 - \left[\frac{OD\ treated\ cells - OD\ medium\ control}{OD\ cell\ control - OD\ medium\ control} \times 100 \right]$$

A checkerboard microplate method was applied to study the effects of interactions between the resistance modifiers methylprototribestin (**2**) and the mixture of the 3 acetylated isomers of methylprotodioscin (**5-7**) and the anticancer agent doxorubicin on cancer cells as an *in vitro* model of combination chemotherapy. The dilutions of doxorubicin (A) were made in a horizontal direction and those of the resistance modifiers (B) vertically in the microtitre plate in a volume of 100 µl. The cell suspension in the tissue culture medium was distributed into each well in 50 µl containing 5×10⁴ cells. The plates were incubated in a CO₂ incubator at 37°C for 48 h. The cell growth rate was determined after MTT staining and the intensity of the blue colour was measured on an Multiscan EX ELISA reader. Drug interactions were evaluated according to the following system:

$$FIC_A = ID_{50A\ combination} / ID_{50A\ alone}$$

$$FIC_B = ID_{50B\ combination} / ID_{50B\ alone}$$

where ID=inhibitory dose and FIC=fractional inhibitory concentration. FIX=FIC_A+FIC_B, where FIX=fractional inhibitory index. FIX<0.5 indicates synergism, 0.5<FIX<1 indicates an additive effect, 1<FIX<2 indicates an indifferent effect, and FIX>2 indicates antagonism between the anticancer drug and the potential MDR modifier.

Octanol/water partition coefficient (Log₁₀ P) calculation. The theoretical Log₁₀ P was determined by using the JME molecular editor (<http://www.molinspiration.com/>).

Results

Various biological activities of saponins and structurally related compounds have been extensively studied and several hundred structures have been elucidated. In our experiments, six pure saponins (**1-4**, **8**, **9**), 3 saponin isomers in a mixture (**5-7**) and 2 phenolics (**10**, **11**) isolated from the saponin fractions of *T. terrestris* and *S. excelsa* were evaluated for their antiproliferative and multidrug resistance inhibitory

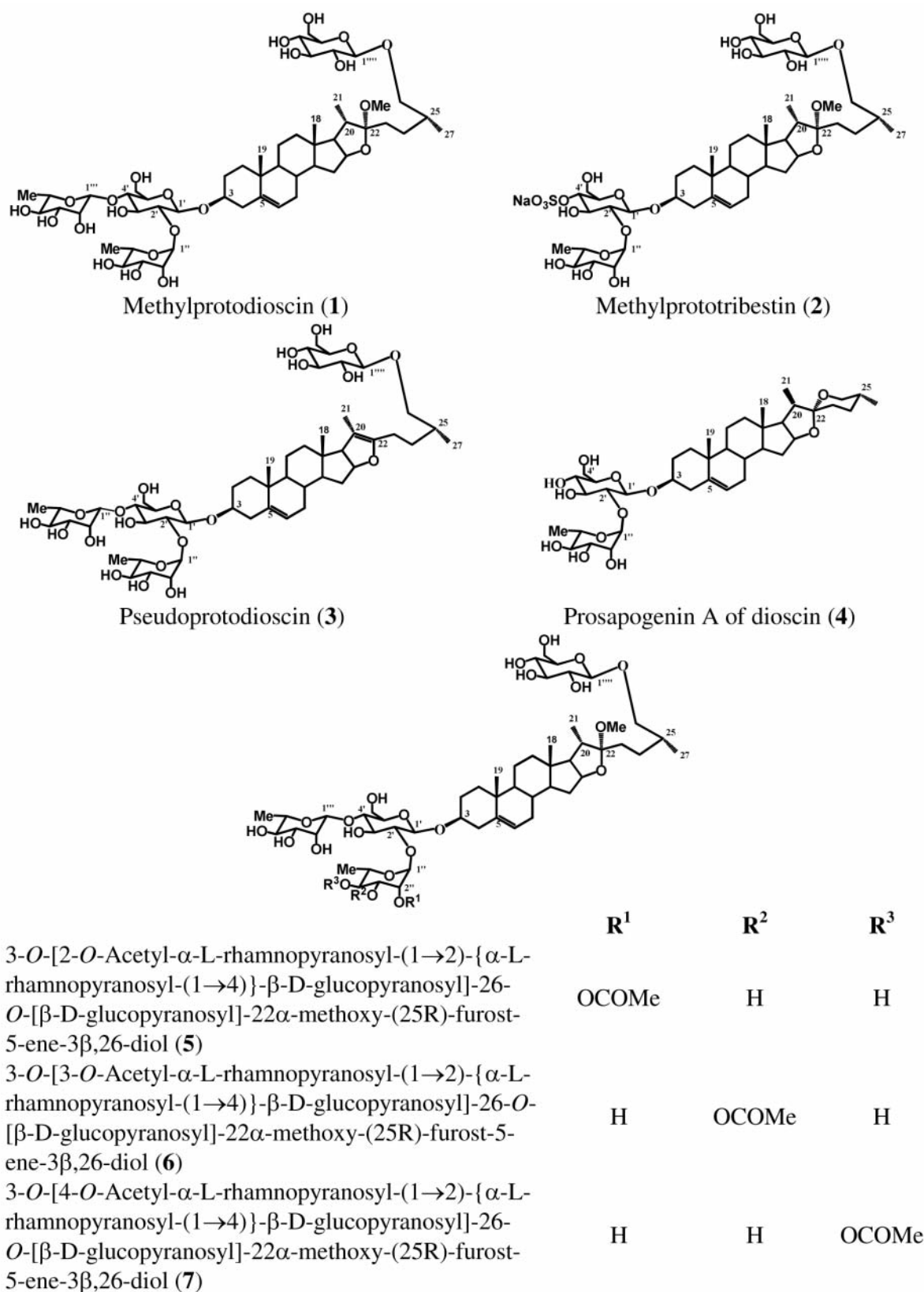


Figure 1. Chemical structures of the compounds (1-7).

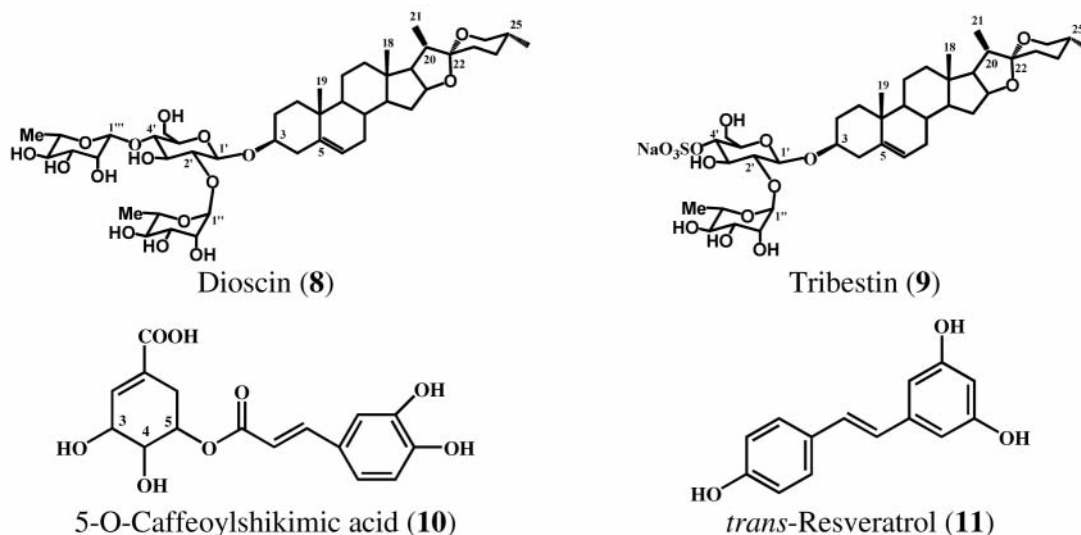


Figure 2. Chemical structures of tested compounds (8-11).

effects on *mdr1*-transfected mouse lymphoma cells. In the antiproliferative experiment, the 50% growth inhibitory dose (ID_{50}) values were found to be between 12.64 and 20.62 $\mu\text{g/ml}$ after incubation at 37°C for 72h in a long-term experiment.

Then the effects of saponins and structurally related compounds isolated from *S. excelsa* and *T. terrestris* were studied on the rhodamine 123 (R123) accumulation on human *mdr1*-transfected mouse lymphoma cells. In this experiment, 1×10^6 cells were exposed to 2 and 20 $\mu\text{g/sample}$ for 10 min before R123 was added to the cells. The toxic effects of the compounds were checked with trypan blue test. Apparently, the compounds shown no toxic effects on 1×10^6 cells at the applied 40 $\mu\text{g/ml}$ concentration during the short incubation period. The majority of the saponin derivatives was ineffective on R123 accumulation when applied in 4 $\mu\text{g/ml}$ concentration, but they were able to reverse multidrug resistance on the mouse lymphoma cells in 40 $\mu\text{g/ml}$ concentration incubated for 30 min. Verapamil was applied as a positive control. The results relating to MDR are shown in Table I.

The MDR reversal effects of the most effective compounds found in the antiproliferative assay were measured in combination with the anticancer drug doxorubicine *in vitro*. The activity of combinations of methylprototribestin (2) (Figure 3 and Table II) and the mixture of 3 acetylated isomers of methylprotodioscin (5-7) (Figure 4 and Table III) with doxorubicin were examined. Synergistic interaction between methylprototribestin (2) and doxorubicin ($FIX=0.498$) was found, and the interaction between the mixture of 3 acetylated isomers of methylprotodioscin (5-7) and doxorubicin ($FIX=0.984$) was

Table I. The effects of compounds isolated from *Smilax excelsa* and *Tribulus terrestris* on rhodamine 123 accumulation in MDR mouse lymphoma cells: dose-response experiments.

Compound	Flow cytometric analysis parameters*		
	Concentration ($\mu\text{g/ml}$)	FL-1 ^a	Fluorescence activity R
Verapamil	10	124.86	10.95
Methylprotodioscin (1)	40	27.87	2.44
Methylprototribestin (2)	40	75.92	6.67
Pseudoprotodioscin (3)	40	47.09	4.13
Prosapogenin A of dioscin (4)	40	32.03	2.80
Mixture of compounds (5-7)	40	68.40	6.00
Dioscin (8)	40	29.41	2.58
Tribestin (9)	40	47.71	4.19
5-O-Caffeoylshikimic acid (10)	40	33.82	2.97
<i>trans</i> -Resveratrol (11)	40	18.13	1.59
DMSO control	1%	17.51	1.53

*The results are from one representative flow cytometric experiment in which 1×10^4 individual cells were investigated. ^aFluorescence intensity.

found to be additive (Figure 4 and Table III). The results reveal that the compounds exhibit synergistic and additive effects in combination with doxorubicin.

Discussion

The emergence of MDR in cancer cells leads to the ineffectiveness of chemotherapy. Various compounds have been investigated for their ability to reverse MDR, including

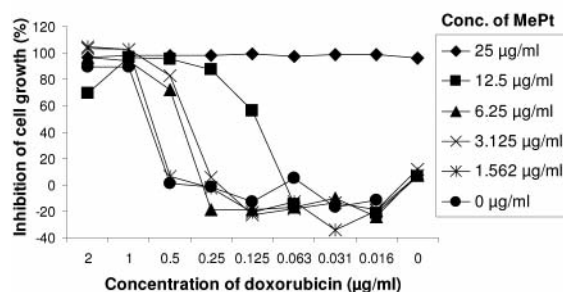


Figure 3. The antiproliferative effect of methylprototribestin (MePt) and doxorubicin in combination.

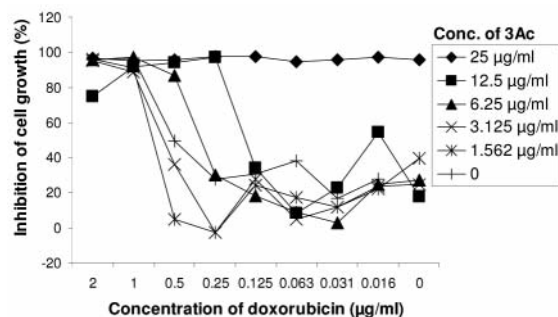


Figure 4. The antiproliferative effect of the mixture of 3 acetylated isomers of methylprotodioscin (3Ac) and doxorubicin in combination.

synthetic (21, 22) and naturally-occurring plant-derived compounds (23, 24).

Saponins are generally found in the roots, flowers and seeds of the higher plants and have long been used against various diseases, to enhance natural resistance, to restore the normal function of the body, to strengthen the constitution (25).

The present study was undertaken to access the inhibition of MDR of cancer cells by saponins and structurally related compounds. The resistance modifier compound **2** exhibited synergy, while compounds **5-7** had only additive effect in combination with doxorubicin, which is a well-known substrate of the P-gp-mediated efflux pumps. The activity of these saponins appears to correlate the position of sulphur-containing substituents, this may refer to the role of charge transfer complex formation in the binding of the compounds to P-gp.

The analysis of the structure activity relationship demonstrated the presence of sulphate and acetyl groups bound to the sugar moieties in compounds **2** and **5-7**. The calculated partition coefficients ($\text{Log}_{10} p=0.09$ and 1.41 for **2** and **5-7**, respectively) indicate some differences in hydrophobicity of these compounds, as well. Obviously, other factors may also interfere with the ability of steroidal furostanol saponins to interact with the P-gp efflux pump.

The *in vitro* experimental data indicate that some of the saponins can be regarded as promising structures for rational drug design of P-gp inhibitors with a novel mechanism of action.

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Table II. Evaluation of antiproliferative effect of methylprototribestin and doxorubicin in combination. *n.a.: non-applicable: in the concentration of 25 µg/ml, the cell proliferation was totally blocked by compound **2**, therefore, the FIX value couldn't be calculated from the curve.

Methylprototribestin (2) (µg/ml)	FIX value
1.562	0.783
3.125	0.477
6.25	0.622
12.5	0.108
25	*n.a.
FIX mean	0.498

Table III. Evaluation of antiproliferative effect of the mixture of 3 acetylated isomers of methylprotodioscin and doxorubicin in combination. *n.a.: non-applicable: in the concentration of 25 µg/ml, the cell proliferation was totally blocked by compounds **5-7**, therefore, the FIX value couldn't be calculated from the curve.

Mixture of 5-7 (µg/ml)	FIX value
1.562	0.976
3.125	1.417
6.25	1.101
12.5	0.441
25	n.a.
FIX mean	0.984

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