layer chromatography (TLC) study was carried out on silica gel plates (Kieselgel 60 $F_{254}$, Merck) using solvent systems n-BuOH/AcOH/$H_2O$ (4:1:1) and $CHCl_3$/MeOH (9:1). The spots were visualized by spraying anysaldehyde/conc. $H_2SO_4$ reagent, followed by heating at 110°C. Chromatography (CC) was carried out with Diaion HP-20 (Supelco) and silica gel 60 (40-60 μm, Merck).

### Plant material

The roots of *G. trichotoma* were collected in August 2008 at the Black Sea coast, Bulgaria. A voucher specimen (SO 103887) was deposited at the Herbarium of the Faculty of Biology, Sofia University.

### Extraction and isolation

Air-dried powdered plant material of *G. trichotoma* (740 g) was exhaustively extracted with 80% methanol. After partial evaporation the aqueous solutions were extracted with $CH_2Cl_2$, EtOAc, and n-BuOH successively. The residue from the n-BuOH layer was separated by CC on a Diaion HP-20 column, using $H_2O$/MeOH (100:0 → 0:100) and further purified by flash chromatography over silica gel with $CH_2Cl_2$/MeOH/$H_2O$ (18:11:1) to yield gypsogenic acid (50 mg) [Figure 1]. The structure of the compound was determined based on the spectral evidence (HRESI-MS, $^1H$ and $^{13}C$ NMR, correlation spectroscopy (COSY), heteronuclear single-quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC) experiments).[11]

### Cell lines and culture conditions

The following human cell lines were used: EJ (urinary bladder carcinoma), SKW-3 (T-cell leukemia), BV-173, K-562 and LAMA-84 (chronic myeloid leukemia), and HL-60 (acute myeloid leukemia) and its resistant variant HL-60/Dox which is characterized by the expression of the multi-drug resistance-associated protein MRP-1. All leukemic cell lines were obtained from DSMZ (Braunschweig, Germany) and EJ cells were obtained from the American Type Culture Collection (Rockville, MD, USA). All cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine (all from Lonza, Belgium) under standard conditions (37°C in an incubator with humidified atmosphere containing 5% $CO_2$). The cell cultures were supplemented with fresh medium two or three times per week to maintain them in log phase. HL-60/Dox cells were maintained in medium containing 0.2 μM doxorubicin in order to sustain their multidrug-resistance (MDR) phenotype. One week prior to cytotoxicity determination however, they were kept in drug-free medium in order to avoid synergistic interaction between doxorubicin and the tested compound.

### MTT assay for cell survival and proliferation

Exponentially growing cells were seeded into 96-well microplates (100 μl/well at a density of $2 \times 10^5$ cells/ml for leukemic cells or $5 \times 10^4$ for EJ cells) and exposed to various concentrations of gypsogenic acid for 72 h. The cell survival fraction was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide) dye reduction assay,[15] performed with some modifications.[16] Briefly, after incubation with the test compound, MTT solution (10 mg/ml in PBS) was added (10 μl/well). Plates were further incubated for 3 h at 37ºC and the formazan crystals formed were dissolved by addition of 110 μl solvent (5% formic acid in 2-propanol) per well and mixing. Absorption was measured by an automated microtiter plate spectrophotometer (Labexim LMR-1, Lengau, Austria) at 550 nm. For each concentration at least four wells were used. Complete medium (100 μl), MTT solution (10 μl) and 5% formic acid in 2-propanol (110 μl) were used as blank solution.

### Statistics and evaluation of cytotoxic effects

Cell survival fractions were calculated as percentages of respective untreated controls, taken for 100%. Using the GraphPad Prism 5.01 program (GraphPad Software, San Diego, California, USA), concentration-effect curves was fitted, which were then used to interpolate respective $IC_{50}$ values and their 95% confidence intervals (CI).

## RESULTS AND DISCUSSION

Fractionation of methanolic extract, obtained from the roots of *G. trichotoma*, by a combination of CC over Diaion HP-20 and silica gel resulted in the isolation of gypsogenic acid.[11]

Gypsogenic acid has previously been shown to possess antibacterial and trypanocidal activity. It inhibited the growth of six cariogenic gram-positive bacterial strains[17] and was active against blood trypomastigote forms of

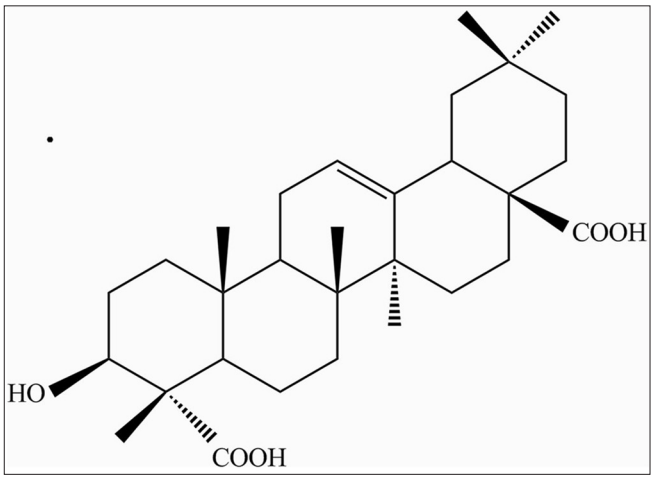


**Figure 1:** Chemical structure of gypsogenic acid

*Trypanosoma cruzi*.[18] Gypsogenic acid was also found to have antihepatotoxic activity in a $CCl_4$-based model of liver injury.[19] In the present study, gypsogenic acid was evaluated for cytotoxic activity against a panel of human tumor cell lines and concentration-dependent cytotoxic effects were observed [Figures 2 and 3]. BV-173 cells were most sensitive ($IC_{50}$ = 41.4 μM; 95% CI: 38.6-44.3 μM), HL-60 cells ranked second ($IC_{50}$ = 61.1 μM; 95%

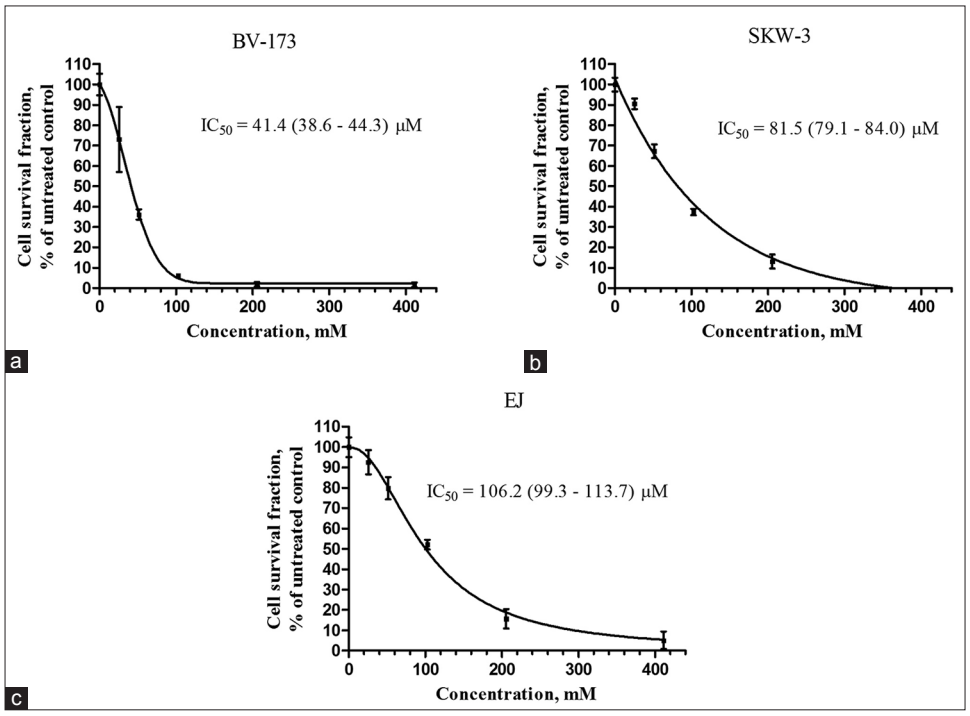


**Figure 2:** Survival of (a) BV-173, (b) SKW-3, and (c) EJ, tumor cells after exposure to gypsogenic acid for 72 h. Cell survival fractions were measured using the MTT dye reduction assay and are given as percentages of the respective untreated controls. Bars denote standard deviation

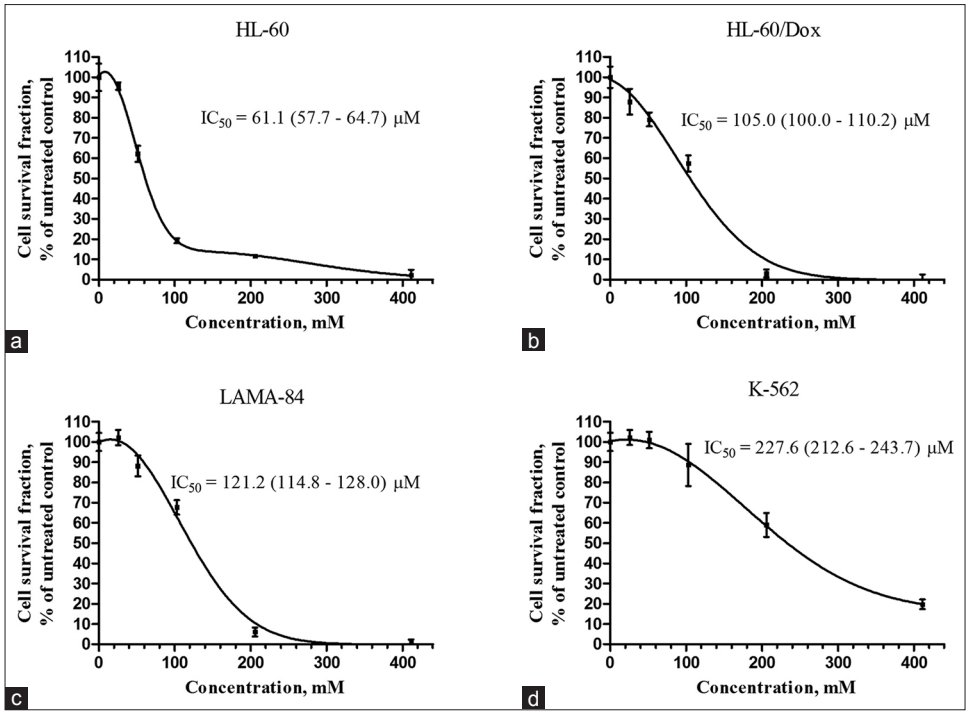


**Figure 3:** Survival of (a) HL-60, (b) HL-60/Dox, (c) LAMA-84, and (d) K-562, tumor cells after exposure to gypsogenic acid for 72 h. Cell survival fractions were measured using the MTT dye reduction assay and are given as percentages of the respective untreated controls. Bars denote standard deviation

CI: 57.7-64.7 μM), and SKW-3 third ($IC_{50}$ = 81.5 μM; 95% CI: 79.1-84.0 μM). Gypsogenic acid had similar efficacy against HL-60/Dox, LAMA-84, and EJ cells with $IC_{50}$ values ranging between 100 and 125 μM. K-562 cells were the least sensitive to gypsogenic acid ($IC_{50}$ = 227.6 μM; 95% CI: 212.6-243.7 μM). Our data for the K-562 cell line are in accordance with those obtained by Lee *et al.*,[2] who studied gypsogenic acid isolated from *A. rossii*, while HL-60 cells were more sensitive in our experiment.

## CONCLUSION

In summary, gypsogenic acid isolated from *G. trichotoma* exhibited moderate cytotoxic activity against leukemic cells with lymphoid (SKW-3 and BV-173) or myeloid phenotype (HL-60, K-562, and LAMA-84), as well as against the EJ bladder carcinoma cell line. Bcr-Abl expressing myeloid cells (LAMA-84 and especially K-562) displayed lower sensitivity. HL-60/Dox cells were less sensitive to gypsogenic acid than the parent cell line, which shows that the compound is probably a substrate of MRP-1.

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