Cytotoxic Activity and Absence of Tumor Growth Stimulation of Standardized Mistletoe Extracts in Human Tumor Models *In Vitro*

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Abstract. *Mistletoe extracts are widely used in complementary and alternative cancer therapy in Europe. The extracts possess cytotoxic, as well as immunostimulatory effects. However, some investigators have suggested that low doses of mistletoe extracts could also induce tumor growth. The mistletoe extracts Helixor A, Helixor M and Helixor P were investigated for growth inhibitory and stimulatory effects in a panel of 38 human tumor cell lines in vitro. Mistletoe lectin I (ML-1), adriamycin and interleukin-6 (IL-6) were used as reference compounds. All three mistletoe preparations showed cytotoxic activity [T/C (Test/Control) <30%]: Helixor P was the most potent, followed by Helixor M and Helixor A with IC_{50} (50% inhibitory concentration) values of 68.4, 114 and 133 μg/ml, respectively. The IC_{50} values of ML-1 and adriamycin were 0.026 and 0.069 μg/ml. None of the human tumor cell lines in the panel showed growth stimulation (T/C (Test/Control) >125%) by the mistletoe extracts or ML-1, apart from two exceptions in the colon carcinoma cell line HCC-2998, in which Helixor M and ML-1 showed a marginal stimulation (T/C 128% and 131%, respectively) at one concentration only. Further investigations into the latter effect of Helixor M and ML-1 in the HCC-2998 line using five different proliferation assays, modified cell culture conditions and the identical production charge of mistletoe extract, as well as a new one, did not confirm the previous observation. It was concluded that the marginal stimulation found in the earlier experiments was a statistical coincidence. Helixor mistletoe preparations and ML-1 have cytotoxic activity and do not stimulate tumor cell proliferation in vitro which is in accordance with previous scientifically based observations on aqueous mistletoe extracts.*

Mistletoe extracts (*Viscum album* L.) have been widely used in complementary and alternative treatment for cancer in Europe for almost nine decades (1, 2). The mistletoe is a semi-parasitic plant that grows on several types of trees, such as pine (Pinus), apple (Malus), oak (Quercus) and spruce (Picea). The extracts contain lectins, viscotoxins, alkaloids and other constituents, but the composition depends upon the host tree, and the extraction and manufacturing processes (3, 4). A variety of commercial mistletoe preparations are available and marketed as injectable prescription drugs. Helixor is a non-fermented aqueous extract that is standardized for its biological effect on human leukemia cells *in vitro* and marketed as Helixor A (from fir trees), Helixor M (from apple trees) and Helixor P (from pine trees) (5).

Mistletoe extracts possess cytotoxic activity against cancer cells *in vitro* (3, 6, 7), can stimulate cells of the immune system to release cytokines *in vitro* and *in vivo* (8-10), and stabilize DNA in white blood cells after exposure to DNA-damaging agents (11). Lectins, of which the major one is mistletoe lectin-1 (ML-1), and viscotoxins have been identified as the main active principles of the mistletoe extracts. Lectins are heterodimeric glycoproteins that belong to the group of type II ribosome-inactivating proteins (12-14). After intracellular uptake of lectins by tumor cells the protein synthesis is inhibited which results in cell death *via* p53-independent apoptosis and/or necrosis, in a concentration-dependent manner (6, 15-17). At low doses (≤1 ng ML/ml or 1 ng ML/kg body weight) lectins show

Abbreviations: ATCC: American Type Culture Collection, Rockville, MD, USA; BrdU: 5-bromo-2'-deoxyuridine; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ECACC: European Collection of Cell Cultures, Salisbury, Wiltshire, UK; FCS: fetal calf serum; h: hours; IC_{50}: 50% inhibitory concentration; IL: interleukin; ML: mistletoe lectin; NCI: National Cancer Institute, Bethesda, MD, USA; PI: propidium iodide; T/C: Test/Control.

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immunomodulatory activity *via* stimulation of effector cells, such as monocytes, macrophages, NK-cells, B- and T-lymphocytes that subsequently release cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor α (15, 18, 19).

Since the early 1990's a few investigators have believed that low doses of ML-1, as well as mistletoe extracts stimulate growth of hematological tumors and other tumor entities which respond to immunological therapy, such as melanoma and renal cell carcinoma (20, 21). For instance, Gabius *et al.* (20) found *in vitro* a slight enhancement of tumor cell growth by ML-1 in the 50-70 pg/ml range in three out of eight human sarcoma and two out of four human melanoma cell lines. However, this tumor growth enhancement could not be confirmed by Thies *et al.* (22), Büssing *et al.* (23), Burger *et al.* (24, 25) or Maier and Fiebig (7) for mistletoe lectins and mistletoe extracts.

In the present study three mistletoe extracts, Helixor A, Helixor M, and Helixor P were evaluated for antiproliferative and/or stimulatory effects *in vitro* in a panel of 38 human tumor cell lines using a cellular proliferation assay. The cell line panel included the five melanoma and sarcoma lines that had been interpreted to enhance growth upon exposure to mistletoe lectins (20).

Materials and Methods

Cell lines. Characteristics of the human tumor cell lines used are shown in Table I. Ten tumor cell lines were derived from the Oncotest human tumor xenograft collection. Establishment and characterization of these cell lines and the origin of the donor xenografts have been described previously (26, 27). Thirteen tumor cell lines were provided by the US National Cancer Institute (Bethesda, MD, USA) and the 15 other tumor cell lines were obtained from the cell banks of ATCC (Rockville, MD, USA), ECACC (Salisbury, UK) and DSMZ (Braunschweig, Germany). The panel included the five tumor cell lines that responded to ML-1 with tumor cell growth as reported by Gabius *et al.* (20), namely the sarcoma lines Hs729, SK-UT-1B and SK-LMS-1 and melanoma lines SK-MEL-28 and HT-144, as well as five further cell lines described in the same paper which did not show growth stimulation to ML-1 (RPMI-8226, CCRF-CEM, HL-60, DU-145 and MALME-3M). Cells were routinely passaged once or twice weekly and were maintained for no longer than 20 passages in culture. All cells were grown at 37˚C in a humidified atmosphere (95% air, 5% CO_2) in RPMI 1640 medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS; PAA) and 0.1 mg/ml gentamicin (PAA).

Cell proliferation assays. The propidium iodide (PI) assay was the standard method for determining the number of viable tumor cells in the proliferation and cytotoxicity experiments. The red-fluorescent PI only penetrates damaged cell membranes and binds to the DNA. The amount of fluorescence correlates with the number of non-vital cells. Four additional colorimetric assays have been used for comparison in further experiments. These assays are either based upon the conversion of a tetrazolium salt by metabolically active cells into a formazan dye (XTT, WST-1 and CellTiter 96 assays) or the

Table I. *Characteristics of the 38-human tumor cell line panel.*

	Tumor type	Cell line	Origin
1	Bladder, transitional cell carcinoma	BCL T24	ATCC
2	Bladder, urothelial adenocarcinoma	BXF 1218L	Xenograft
3	Colon, colorectal adenocarcinoma	CCL DLD-1	NCI
4	Colon, adenocarcinoma	CCL HCC-2998	NCI
5	Colon, colorectal carcinoma	CCL HCT-116	NCI
6	Colon, carcinoma	CCL HT-29	NCI
7	Leukemia, acute lymphoblastic	LECL CCRF-CEM	NCI
8	Leukemia, promyelocytic	LECL HL60	NCI
9	Leukemia, T-cell lymphoblast	LECL Jurkat	DSMZ
10	Lung, large cell	LCL NCI-H460	NCI
11	Lung, adenocarcinoma	LXFA 526L	Xenograft
12	Lung, adenocarcinoma	LXFA 629L	Xenograft
13	Lung, large cell	LXFL 529L	Xenograft
14	Lymphoma, Burkitt's	LYCL Daudi	ATCC
15	Lymphoma, T-cell	LYCL HUT-78	ECACC
16	Lymphoma, Burkitt's	LYCL Raji	ATCC
17	Lymphoma, histiocytic	LYCL U-937	ATCC
18	Mammary, adenocarcinoma	MACL MCF7	NCI
19	Mammary, adenocarcinoma	MACL MDA-MB-231	ATCC
20	Mammary, adenocarcinoma	MACL MDA-MB-468	ATCC
21	Mammary, adenocarcinoma	MAXF 401NL	Xenograft
22	Melanoma, malignant	MECL HT-144	ATCC
23	Melanoma, malignant	MECL Malme-3M	NCI
24	Melanoma, malignant	MECL SK-MEL-28	NCI
25	Plasma cell leukemia	MMCL L-363	DSMZ
26	Myeloma, multiple	MMCL NCI-H929	DSMZ
27	Myeloma, multiple	MMCL RPMI-8226	NCI
28	Ovarian, adenocarcinoma	OVXF 1619L	Xenograft
29	Ovarian, adenocarcinoma	OVXF 899L	Xenograft
30	Pancreas, epithelioid carcinoma	PACL PANC-1	ATCC
31	Pancreas, adenocarcinoma	PAXF 1657L	Xenograft
32	Prostate, carcinoma	PRCL DU-145	NCI
33	Prostate, adenocarcinoma	PRCL PC-3M	NCI
34	Renal, hypernephroma	RXF 486L	Xenograft
35	Renal, hypernephroma	RXF 944L	Xenograft
36	Sarcoma, rhabdomyosarcoma	SCL Hs729.T	ATCC
37	Sarcoma, leiomyosarcoma	SCL SK-LMS-1	ATCC
38	Uterus, mesodermal tumor (mixed)	UCL SK-UT-1B	ATCC

ATCC: American Type Culture Collection, Rockville, MD, USA; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ECACC: European Collection of Culture Cells, Salisbury, Wiltshire, UK; NCI: National Cancer Institute, Bethesda, MD, USA.

incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the DNA of dividing cells in place of thymidine.

Propidium iodide (PI). A modified PI assay (28) was used to assess the effects of the Helixor mistletoe extracts (A, P, M), ML-1 and IL-6 on the growth of the human tumor cell lines.

Briefly, cells were harvested from exponentially growing cultures by trypsinization (not for cells growing in suspension), counted and plated in 96-well flat-bottomed microtiter plates at a cell density of 4,000-10,000 viable cells/well, dependent upon the cell line. After 24 h recovery to allow the cells to resume exponential growth, 10 µl of culture medium (six control wells per plate) or culture medium containing the test compounds were added to the wells. Each concentration was plated in triplicate. Mistletoe extracts were applied at five concentrations with one log increments (0.015-0.15-1.5-15-150 µg/ml total plant extract). The final concentrations of ML-1 and IL-6 ranged from 0.01 to 100 ng/ml, and adriamycin was used from 0.03 to 300 ng/ml. Following four days of continuous drug exposure, cell culture medium without or with test compounds was aspirated and replaced by 200 µl of an aqueous PI solution (7 µg/ml). Since PI only passes leaky or lysed cell membranes, the DNA of dead cells would be stained and measured, while living cells would not be stained. To measure the proportion of living cells, cells were permeabilised by freezing the plates, resulting in death of all cells. After thawing of the plates fluorescence was measured using the Cytofluor 4000 microplate reader (excitation 530 nm/emission 620 nm), giving a direct relationship to the total cell number. The assay included untreated and positive controls (adriamycin).

Growth inhibition/stimulation was expressed as test/control x 100 (%T/C) as the median of 3 independent experiments. Tumor growth stimulation was defined as T/C >125% and cytotoxic activity was defined as T/C <30%.

Assays were only considered evaluable if the positive control (adriamycin) induced a T/C value of <30% and if untreated control cells had a fluorescence intensity > 500 units.

XTT, WST-1 and CellTiter96® proliferation assays. The XTT, WST-1 and the CellTiter96® proliferation assays were carried out using the Cell Proliferation Kit II (Roche, Penzberg, Germany), the Cell Proliferation Reagent WST-1 Kit (Roche) and the CellTiter 96® Aqueous One Solution Cell Proliferation Kit (Promega Corporation, Madison, WI, USA), respectively. The assays were performed according the instructions of the manufacturers.

After the four days continuous exposure to the test compounds 75 µl of the XTT labeling mixture (50:1 mixture of XTT labeling reagent and electron coupling reagent), 15 µl of the WST-1 cell proliferation reagent or 30 µl of the CellTiter 96® aqueous one solution reagent was added to each well and plates were incubated for 4 h in a humidified atmosphere at 37˚C and 5% CO_2. The amount of formazan formed was quantified spectrophotometrically by an ELISA reader at 450 nm (490 nm for CT96) and the reference wavelength at 650 nm. Untreated control cells were represented by 12 wells per plate, treated groups by six wells per concentration.

BrdU assay. The BrdU assay was performed according to the instructions of the manufacturer of the kit (Roche). It is a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis. The incorporated BrdU is detected by the anti-BrdU-POD working solution.

After four days continuous exposure to the test substances 20 µl of the BrdU labeling solution was added to each well and plates were incubated for 18 h in a humidified atmosphere at 37˚C and 5% CO_2. The labeling medium was removed, the cells fixed and the DNA denatured using 200 µl FixDenat solution for 30 min at room temperature. After removal of the FixDenat solution the incorporated BrdU was detected by adding anti-BrdU-POD conjugate (100 µl/well) for 90 min. The antibody conjugate was removed and the wells rinsed 3x with 200 µl washing solution per well. Finally, the substrate solution was added (100 µl/well) and incubated for 5-30 min to allow color development for photometric detection. After adding 25 µl H_2SO_4-stop-solution absorbance was measured at 450 nm and the reference wavelength at 650 nm using an ELISA reader (Dynatech MR5000). Here also, untreated control cells were represented by 12 wells per plate and treated groups by six wells per plate.

Data evaluation and statistical analysis. Three independent experiments were performed for each test compound and each test concentration in the PI assay. In all experiments individual data were determined in triplicate (treated groups) and in sextuplicate (untreated groups). In all other assays treated groups were determined in sextuplicate and untreated groups were represented by 12 data points per experiment. For each experiment, individual data for the treated groups were normalized with respect to the mean values for the untreated group of the same experiment (T/C values). In the case of normal distributed values Student's two-tailed *t*-test was used and when variances differed substantially the Mann-Whitney rank sum test was applied. Differences between the values for treated and untreated cells were considered as significant at a global significance level of $p<0.05$ with Bonferroni-Holm correction for multiple testing. Statistical analysis was performed using SigmaStat for Windows Version 30.0.1.

Test compounds. The clinical preparations of the mistletoe extracts Helixor A (lot 040263), Helixor M (lot 030954) and Helixor P (lot 030902) were used (Helixor Heilmittel GmbH&CoKG, Rosenfeld, Germany). The ampoules of each of the extracts contained 50 mg total plant extract of *Viscum album* L. in 1 ml water. ML-1 was supplied by the Institut für Phytochemie, Private Universität Witten/Herdecke (Germany), adriamycin (Medac, Hamburg, Germany) was obtained from the local pharmacy (Freiburg, Germany), and the recombinant human IL-6 (# 206-IL) was supplied by R&D Systems (Wiesbaden, Germany). The material was stored at –20˚C.

For comparison two different production batches of Helixor M (lot 030954 and 050152) were used in the follow-up experiments.

The amount of total ML/ml of the Helixor extracts was as follows: Helixor A, 390.6 ng/ml (2.6 ng/ml ML-1, 388 ng/ml ML-3); Helixor P, 1199 ng/ml (0 ng/ml ML-1, 1199 ng/ml ML-3); Helixor M lot 030954, 499.7 ng/ml (22.7 ng/ml ML-1, 477 ng/ml ML-3), and lot 050152, 283 ng/ml (16 ng/ml ML-1, 267 ng/ml ML-3).

Results

Among the three mistletoe extracts Helixor P effected the highest cytotoxic activity in the human tumor cell line panel, followed by Helixor M and Helixor A. At the highest concentration (150 µg/ml) Helixor P was active (T/C <30%) in 15/38 cell lines, Helixor M in 6/38 and Helixor A in 4/38

Table II. *Mean IC_{50} and IC_{70} values of Helixor preparations in the 38-human tumor cell line panel.*

Extract/compound	Mean IC_{50} (μg/ml)	Mean IC_{70} (μg/ml)
Helixor P	68.4	112.0
Helixor M	114.0	144.0
Helixor A	133.0	>150.0
ML-1	0.026	0.054
Adriamycin	0.069	0.280

cell lines. In addition, Helixor P was active at 15 μg/ml in 4/38 cell lines, whereas the other Helixor extracts did not show activity at this concentration. At the concentrations of 0.015-1.5 μg/ml the extracts did not show cytotoxicity. ML-1 was active at the concentrations of 10 and 100 ng/ml in 6/38 and 20/38 tumor cell lines, respectively, and adriamycin at 30 and 300 ng/ml in 2/38 and 19/38 tumor cell lines. IL-6 did not possess cytotoxic activity but enhanced the tumor growth (T/C >125%) in several lines (see below). Regarding overall potency in terms of IC_{50} (50% inhibitory concentration) and IC_{70} values the same order was seen (Table II): ML-1 as the most potent, followed by adriamycin, Helixor P, Helixor M and Helixor A. The order of the cytotoxic activity of the Helixor extracts correlated with their content of total ML/ml.

The IC_{70} profile of ML-1 demonstrated a pronounced selectivity (individual IC_{70} <1/3 mean IC_{70}) for all three leukemia lines (CCRF-CEM, HL60, Jurkat), the mammary cancer MAXF 401NL, the ovarian cancer OVXF 1619L and prostate cancer DU145 (Figure 1A). Helixor P had also a pronounced selectivity for 2/3 leukemia lines, the mammary cancer MAXF 401NL and myeloma L-363 (Figure 1B). Helixor M and A showed the same pattern as Helixor P, but less pronounced. Adriamycin had a different IC_{70} profile: colon cancer line HCT 116, lung cancer line H460, myeloma RPMI-8226, prostate cancer DU 145 and uterus cancer SK-UT-1B were the most sensitive lines (Figure 1C).

Regarding possible growth stimulation of the tumor cell lines in the panel by Helixor extracts and ML-1, it was evident that, apart from two exceptions, none of the T/C values at all concentrations tested of Helixor P, M, A and ML-1 reached the threshold of >125%. This also included the tumor cell lines previously reported by Gabius *et al.* (20) to have enhanced growth. The exceptions were observed in the mistletoe-resistant colon cancer cell line HCC-2998, in which Helixor M showed a T/C value of 128% at the concentration of 15 μg/ml ($p<0.05$) and ML-1 a T/C of 131% at 0.01 μg/ml ($p<0.05$). The remaining Helixor M and ML-1 concentrations did not show T/C values >125%.

These results are in contrast to IL-6 that induced statistically ($p<0.05$) enhanced tumor growth in six tumor cell lines. IL-6 stimulated growth was in most cases concentration-dependent, exhibiting T/C values up to 167% (Figure 2). It concerned 4/10 hematological tumor cell lines (myeloma NCI-H929, leukemia CCRF-CEM, lymphomas HUT-78 and U-937) and 2/28 cell lines derived from solid tumors (lung LXFA 526L, renal RXF 944L).

Table III. *Effect of FCS content of culture medium and prolongation of the incubation period on proliferation of HCC-2998 cells with Helixor M (lot 030954).*

FCS%	T/C% at Helixor M concentration (μg/ml)				
	0.015	0.15	1.5	15	150
Previous experiment					
4 days drug exposure					
10% FCS	95	104	117	128	121
4 days drug exposure					
10% FCS	96	104	96	108	105
5% FCS	96	108	102	103	107
2% FCS	92	99	96	106	95
1% FCS	95	88	107	113	99
7 days drug exposure					
10% FCS	93	105	103	101	96

Follow-up experiments on the colon cancer cell line HCC-2998. The data described above showed that there was some evidence of tumor growth stimulation by one of the three Helixor mistletoe extracts and ML-1 in 37 out of 38 tumor cell lines studied. Only the growth of colon carcinoma HCC-2998 cells was marginally enhanced by Helixor M and ML-1, however each at a different concentration of lectins. In order to investigate whether this stimulatory effect was reproducible or an artifact Helixor M was tested again in HCC-2998 with the same PI assay and, additionally, four other proliferation assays. Two different production batches of Helixor M (lot 030954 [previous batch] and 050152 [new batch]) were used as well as ML-1 and adriamycin.

The repeat of the experiments in the PI assay was performed as described before. Three independent experiments were performed applying each test concentration in triplicate. The stimulatory effect of Helixor M in the previous experiments (T/C 128% at 15 μg/ml) was not confirmed (Table III). The observed marginal enhancement in growth of up to 108% (at 15 μg/ml) was statistically not significant compared to untreated control. This was found for both production batches of Helixor M as well as for ML-1 (data not shown). As in the previous experiments, Helixor M and ML-1 did not show cytotoxic activity in the HCC-2998 tumor cell line at all concentrations tested (0.015-150 μg/ml). Increasing the concentration of Helixor M from 150 μg/ml *via* 500, 1500 to 5000 μg/ml revealed only a slight inhibition of

A. ML-1.

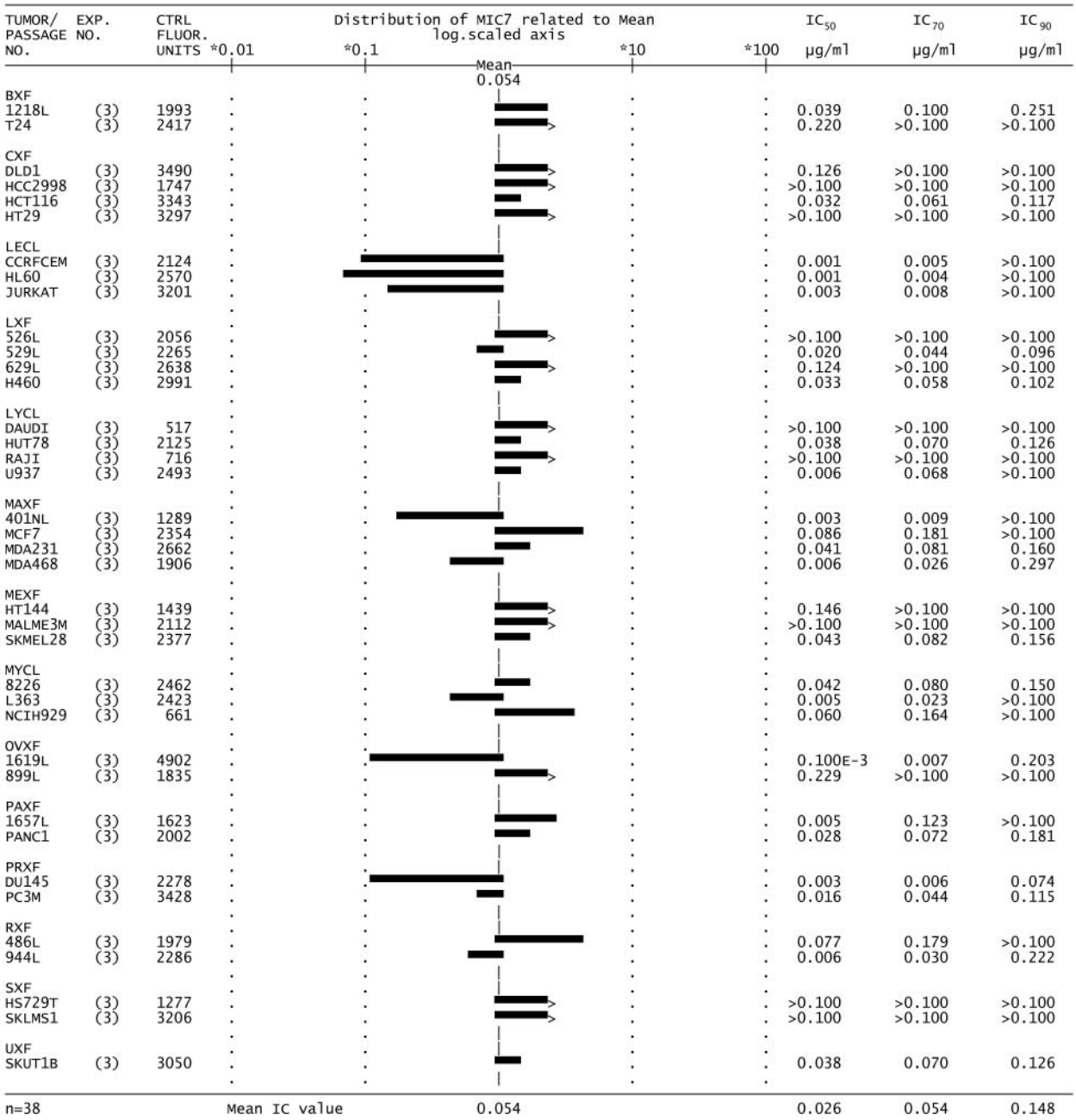


Figure 1. *continued*

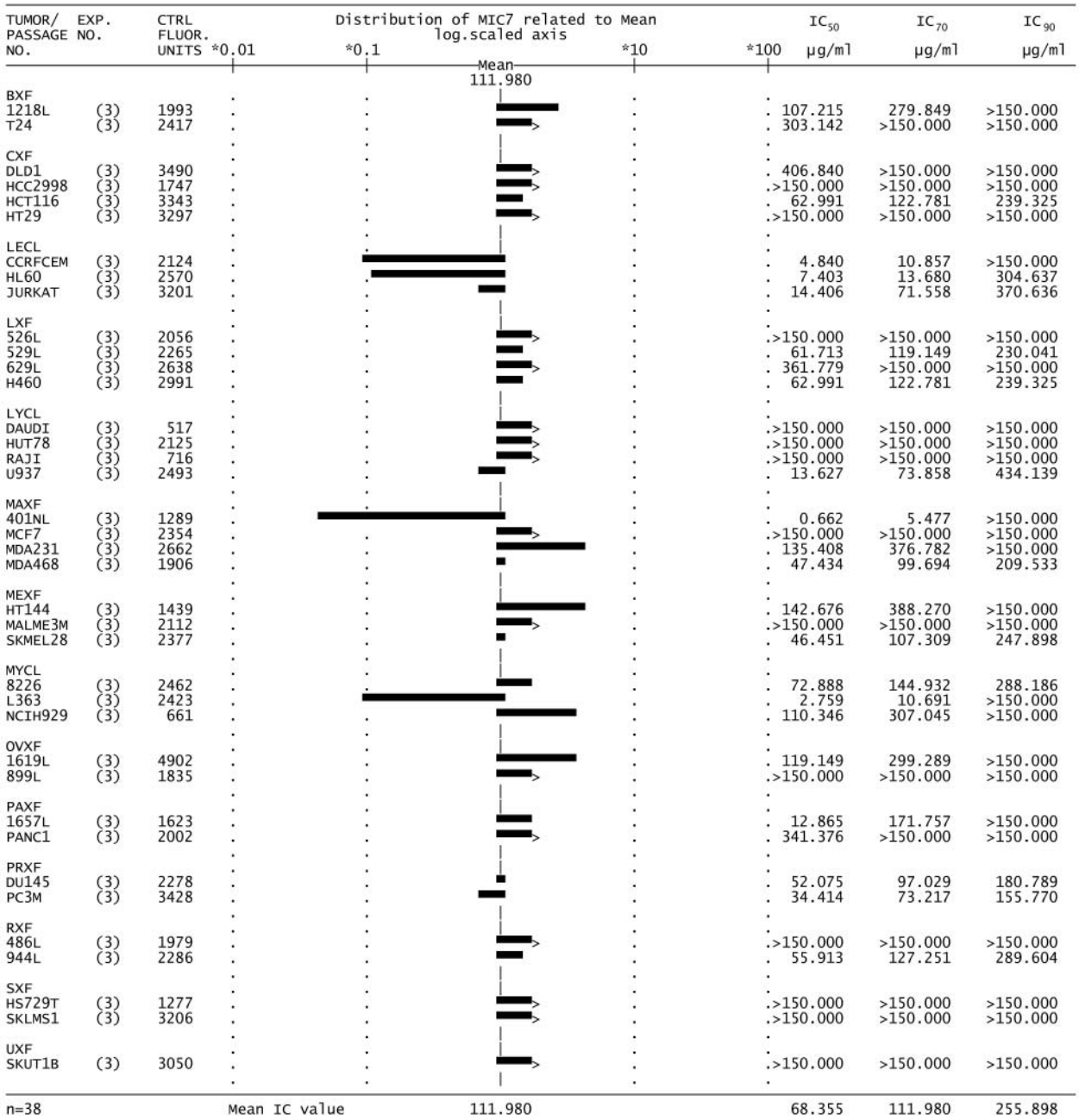
cell growth at the highest concentration tested (5000 µg/ml); the T/C values decreased from the 110-98% range at the lower concentrations (50-1500 µg/ml) to 54% (lot 030954) and 82% (lot 050152) at 5000 µg/ml.

Modifications of the standard culture conditions by changing the amount of FCS in the culture medium [as the presence of FCS may inhibit activity of mistletoe extracts (29)] from 10% to 5%, 2% and 1% FCS had no effect on the activity of both batches of Helixor M neither did the prolongation of the drug exposure time from 4 to 7 days (Table III). Identical results were found for ML-1.

Two Helixor M batches, ML-1 and adriamycin were also investigated in four different proliferation assays: the XTT, WST-1, CellTiter 96® assays and the BrdU assay. Figure 3

Figure 1. *continued*

B. Helixor P.



reveals that none of the test compounds induced stimulation of cell proliferation. Similarly to the results in the PI Assay, inhibition of growth was also not detectable for the mistletoe extract and ML-1 in the applied concentration ranges. In contrast, adriamycin effected cytotoxic activity at the highest test concentration of 3 µg/ml (T/C<20%) detected with all four assays. The experiments with the XTT and WST-1 assays were carried out twice and the CellTiter 96® and BrdU assays three times, similar results were obtained in all runs.

Overall, the earlier observed stimulation of HCC-2998 cells by Helixor M and ML-1 in the PI assay could not be confirmed. Furthermore, investigation of the effect of the mistletoe extract under modified cell culture conditions, or by using different proliferation assays did not show tumor growth stimulation.

Figure 1. *continued*

C. Adriamycin.

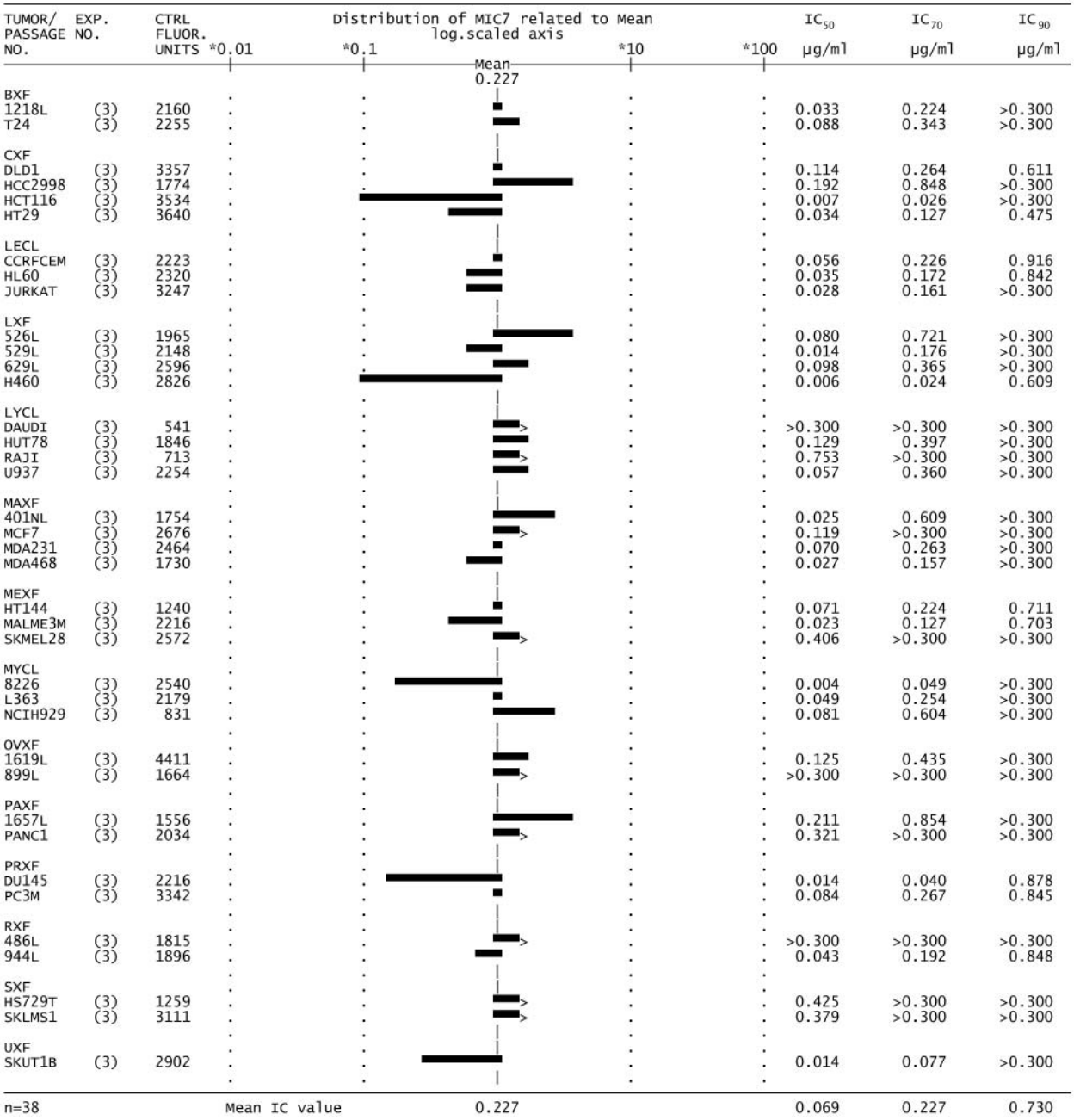


Figure 1. *IC_{70} profiles of ML-1 (A), Helixor P (B) and Adriamycin (C) in a panel of 38 human tumor cell lines. The distribution of the IC_{70} values of the individual tumor cell lines is related to the mean IC_{70} value of the test compound. Variations of individual IC_{70} values from the mean IC_{70} value are expressed as bars in the logarithmically scaled axis. Bars to the left express IC_{70} values lower than the mean IC_{70} (sensitive cell lines), bars to the right show higher individual IC_{70} values (resistant cell lines).*

Discussion

Our experimental data revealed that Helixor mistletoe extracts and ML-1 have: a) cytotoxic activity in the 15-150 µg/ml (equivalent to 0.1-4 ng ML/ml) and 10-100 ng/ml range, respectively; b) each has its own cytotoxicity profile; and, c) they did not stimulate growth of human tumor cells derived from hematological malignancies and solid tumors

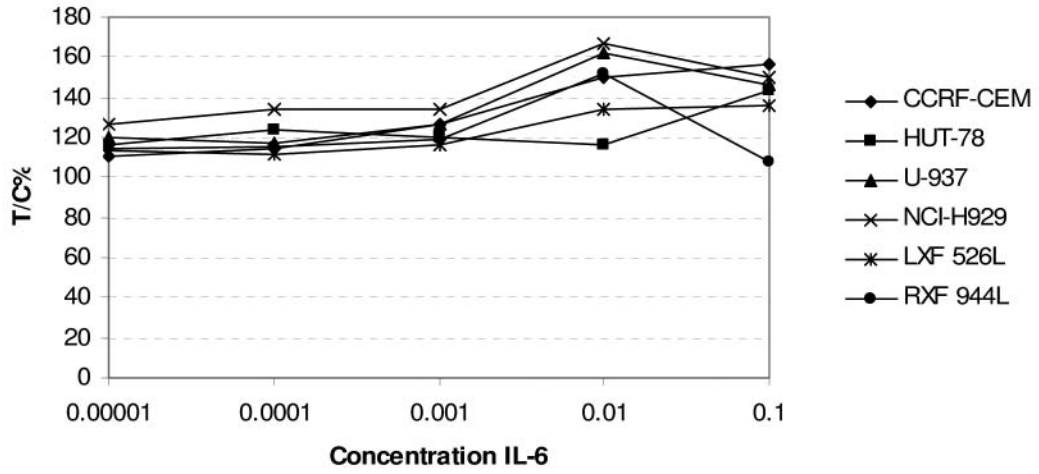


Figure 2. *In vitro concentration-dependent growth stimulation of IL-6 in six out of 38 human tumor cell lines. Growth is expressed as treated/control x100% (T/C%). Results were presented as the mean of three independent experiments. The threshold for growth stimulation was T/C >125%. NCI-H929 (myeloma), U-937 (histiocytic lymphoma), CCRF-CEM (acute lymphoblastic leukemia), HUT-78 (T cell lymphoma), HCC-2998 (colon), LXF 526L (lung cancer) and RXF 944L (renal cancer).*

in vitro in a broad concentration range (15 ng/ml – 150 μg/ml total plant extract, equivalent to approximately 0.1 pg ML/ml – 4 ng ML/ml; ML-1: 10 pg/ml - 100 ng/ml). These data are in agreement with results of other mistletoe extracts obtained in this laboratory (7, 24, 25) and those of others (22, 23, 30).

The absence of stimulation in the tumor cell lines does not mean that the highest T/C value could not be over 100%. The threshold for stimulation was defined as T/C >125%. The T/C values of our experiments are the median of three independent experiments and, depending on the assay, in each experiment each concentration of the drugs was tested three or six times, both to reduce the variability of the experiments. To come to a robust evaluation of the experimental data, they were thoroughly analyzed. First the data of the various groups of each experiment were statistically tested for skewness and depending upon the outcome the appropriate test (Student's *t*-test or Mann-Whitney rank sum test) was used to identify statistical differences between the groups. Moreover, to avoid statistically significant results by chance the Bonferroni-Holm correction for multiple testing was then applied.

Our data also showed that four hematological and two solid tumor cell lines proliferated in response to IL-6 in a dose-dependent pattern. These six tumor cell lines did not respond with proliferation to exposure to the three Helixor extracts and ML-1. The only tumor cell line that showed proliferation to a mistletoe extract and ML-1 was colon HCC-2998, which was mistletoe- and IL-6-resistant, and then at one concentration only (15 μg/ml for Helixor M and 0.01 μg/ml for ML-1). To confirm these surprising results, the experiments with Helixor M and ML-1 were again repeated three times with the standard PI assay. Furthermore, two production batches of Helixor M were used; the one that was already used in the earlier experiments (lot 030954) and a new one (lot 050152). In addition, the standard conditions of the PI assay were modified by varying FCS concentrations, drug exposure time and the dose levels of both test substances. None of these modifications resulted in any growth stimulation of HCC-2998 tumor cells by Helixor M and ML-1.

To corroborate the absence of the stimulation of HCC-2998 tumor cells in the presence of Helixor M and ML-1 observed in the PI assay, the experiments were repeated using four other proliferation assays. Three of the additional proliferation assays (XTT, WST-1 and CellTiter 96®) required the presence of metabolically active cells to convert the substrate into a soluble dye that can be detected by an Elisa reader. The sensitivity of the assays is similar and there are small differences in the performance, for instance the WST-1 assay has a wider linear range and accelerated color development compared to the XTT assay (according to the data of the manufacturer). The fourth assay required the presence of proliferating cells to incorporate BrdU into the DNA. The outcome in all these assays with their different mechanism of action was clear: no tumor growth stimulation. This and the rigorous statistical methods applied in our studies demonstrate that the two cases of stimulation in the HCC-2998 tumor cell line by Helixor M and ML-1, each at one concentration only, was a methodological artifact. This fits with the literature showing that mistletoe extracts, also at low doses, do not induce proliferation of tumor cells, neither from hematological nor from solid tumor origin. Furthermore, ML-1 does not induce proliferation of haematopoietic

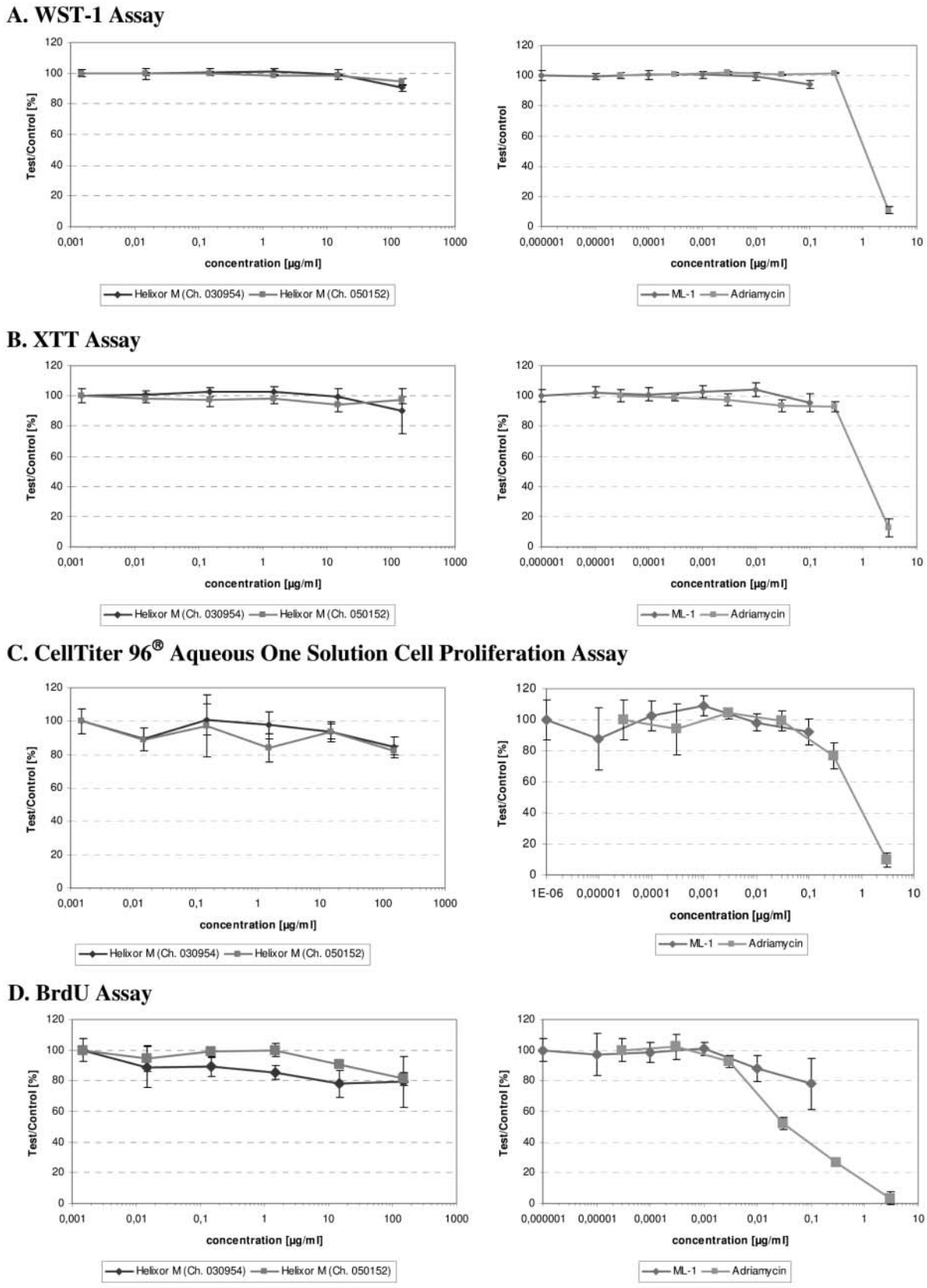


Figure 3. *Effect on cell growth of HCC-2998 cells of Helixor M (two batches), ML-1 and adriamycin, as determined in various proliferation assays in vitro.*

progenitor cells *in vitro*, but enhances proliferation when combined with cytokines like erythropoietin (31). The *in vitro* data presented by Gabius *et al.* (20) are unique in the literature showing stimulation of tumor cells, in most cases at one time-point and at one concentration only without a clear dose- or time-dependency. His statistical and laboratory methods have been questioned (22, 32) and the outcome of his experiments has not been confirmed by our and other laboratories (7, 22-25). We have, thus, shown that the occasional observation of tumor cell proliferation by a mistletoe extract and ML-1 in the PI assay could not be proven by repeating the experiments and applying other proliferation assays. The final conclusion is that the three Helixor mistletoe extracts have cytotoxic activity, each with their own profile, and that they do not stimulate growth of tumor cells *in vitro* over a broad concentration range.

The *in vivo* studies with mistletoe lectin I on increased tumor growth are summarized in the work of Kunze *et al.* (33). In their experimental study the effects of *Viscum album* extract on *N*-butyl-*N*-(4-hydroxy-butyl)-nitrosamine (BBN)-induced carcinogenesis in the urinary bladder of rats was examined. The data of this extended animal trial were used by Gabius as the scientific-based-evidence for the lack of antitumor efficacy, as well as the potential danger for tumor cell proliferation. In contradiction more than 70 animal trials on tumor control, tumor regression, survival rate, anticarcinogenetic activity, bone marrow regeneration, as well as anti-metastatic activity by mistletoe application have been published showing beneficial effects for the majority of investigations (34).

In this context a second publication has also been cited frequently (35). In this randomized phase III adjuvant trial the efficacy and toxicity of low dose recombinant interferon alpha 2 b or recombinant interferon gamma in comparison with an untreated control group as well as a fourth arm with a mistletoe extract was evaluated. A statistically significant clinical benefit for the adjuvant treatment with low dose recombinant interferon alpha 2 b or recombinant interferon gamma or with mistletoe extract in high-risk melanoma patients could not be shown (35).

In summary, recent scientific literature does not support evidence for a proven danger for patients receiving complementary mistletoe therapy.

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