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Aqueous ethanolic extract of St. John's wort (*Hypericum perforatum* L.) induces growth inhibition and apoptosis in human malignant cells *in vitro*

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Extracts of *Hypericum perforatum* (St. John's wort) are widely and effectively used in the treatment of mild to moderate depression. In addition, hypericin, a component of *Hypericum p.* extracts, exhibits light-dependent phototoxic properties and can be used in phototherapy. We therefore investigated the cytotoxic activity of two total *Hypericum p.* extracts, namely from fresh and dried plants in the dark and after exposure to 7.5 J/cm^2 white light illumination and compared it with the effect of hypericin on K562, U937, LN229 glioblastoma cell lines and normal human astrocytes. The chemical toxicity of non-illuminated *Hypericum p.* extracts in the cells tested is low as expressed by a LC_{50} between 1.9–4.1 mg/ml, which corresponds to 10.3–17.3 μM hypericin and 114.4–190.7 μM hyperforin after 48 h treatment. *Hypericum p.* extracts induced dose-dependent growth arrest of human malignant cells in the absence of illumination with GI_{50} values between 0.43–1.77 mg/ml (2.3–9.7 μM hypericin, 26.1–106.7 μM hyperforin) for the fresh plant extract and 0.59–3.03 mg/ml (2.5–12.8 μM hypericin, 24.2–124.7 μM hyperforin) for the dried extract. The growth inhibitory effect of fresh *Hypericum p.* extract was more pronounced in leukemia cell lines K562 and U937, the GI_{50} concentrations being about 7-fold lower than the corresponding LC_{50} for the cell lines K562 and U937, but almost the same as the LC_{50} for LN229 and NHA cells. GI_{50} (μg/ml) for tumor cell lines K562 and U937 (432 and 799) established after 48 h differed significantly ($p < 0.05$) from those of LN229 and normal human astrocytes (1767 and 2900). The light-exposed extracts were more toxic, their LC_{50} and GI_{50} values were reduced to values corresponding to LC_{50} concentration of 3.7–7.4 μM and a GI_{50} of 1.3–3.5 μM for phototoxic hypericin. After exposure to light, there was a significant difference ($p = 0.006$) between the GI_{50} of glioblastoma LN229 cells (582 μg/ml) and normal human astrocytes (1050 μg/ml). Morphological examination by light microscopy and phosphaditylserine exposure on the outer plasma membrane investigated by AnnexinV-binding with flow cytometry after 24 h confirmed that *Hypericum p.* extracts caused apoptosis of treated cells without exposure to light. *Hypericum p.* extracts derived from fresh herbs and from dried herbs which differ in their levels of phloroglucinols (hyperforin and adhyperforin) were compared. The hyperforin content of fresh St. John's wort extract exceeded that of dried plant extract by 47% and the GI_{50} values of fresh plant extract were 73%, 77% and 58% of those established for dried extract in the three malignant cell lines K562, U937 and LN229 in the dark ($p < 0.05$). Under white light (7.5 J/cm^2), both extracts exerted comparable growth inhibitory and apoptosis inducing effects due to the phototoxicity of hypericin, the corresponding concentrations of which were in the range of 1.3–3.5 μM. The data reported in this study suggest that illumination is not essential for the growth inhibitory and apoptotic effects of *Hypericum p.* extracts, but light activation potentiates them. Furthermore, the constituent hyperforin is at least partly responsible for these effects in the dark.

1. Introduction

Hypericum perforatum L. (St. John's wort), has tradionally been used for the treatment of skin injuries, burns, and neuralgia. Recently it has also gained reputation as an effective treatment for mild to moderate depression [1–3]. Many pharmacological studies and animal tests with total extracts and/or their constituents hypericin, flavonoids and more recently hyperforin also support its antidepressant activity [4–10]. The *Hypericum p.* extracts contain a number of constituents, which can be divided into three main groups: naphthodianthrones, phloroglucinols and flavonoids. All three groups are known as biologically active agents [11]. Hypericin is a natural photosensitizing naphthodianthrone which has been shown to cause hypericism in grazing animals feeding on hypericin-containing plants [12, 13]. It has been shown that light-activated hypericin is cytotoxic, inducing apoptosis *in vitro* in various human malignant cells such as fibroblasts [14], mammary carcinoma cells [15], leukemia cells HL-60 and K562 [16], and malignant glioma cells [17, 18]. One of the most widely distributed bioflavonoids in the plant kingdom, quercetin, another constituent of *Hypericum p.* extracts, effectively inhibits the growth of various malignant cells such as K562, MOLT-4, Raji and MCAS cystoadenocarcinoma ovary cell line [19], colon carcinoma cell lines [20] and estrogen-receptor positive (MCF-7) and negative (MDA-MB231) breast cancer cells [21]. Studies on the cytotoxicity of *Hypericum p.* extracts and the component hyperforin – a phloroglucinol derivate – are limited. Recently, it was reported [22], that *Hypericum* extract has a phototoxic effect on human keratinocytes. In addition, *Hypericum p.* extract and the metabolite hyperforin have an inhibitory effect on the peripheral mononuclear cells (PBMC) stimulated with phytohaemagglutinin [23].

Apoptosis (programmed cell death) is an essential regulatory mechanism in the physiological process of cells and plays a central role in development, tissue homeostasis, and thymic selection, as well as in pathologies ranging from neurodegenerative diseases, autoimmune disorders, and viral infection, to cancer [24–26]. Apoptosis is widely used by multicellular organisms to remove unwanted cells. Whereas cell division and apoptosis are usually in balance, disturbances in cell proliferation and in the rate of apoptosis play an important role in the formation of neoplastic lesions [27–29]. Therefore, natural compounds which can influence the balance between cell division and apoptosis are of growing interest. Consequently, the present study was designed to investigate the antiproliferative and apoptotic activity of aqueous alcoholic extracts of *Hypericum p.* on human malignant cells. This report describes the cytotoxic effects of *Hypericum p.* ex-

tracts towards K562, U937, LN229 glioblastoma cell lines and normal human astrocytes (NHA) and provides evidence that *Hypericum p.* extract-induced cytotoxicity correlates with morphologic changes and with phosphatidylserine exposure on the cell membrane typical for apoptotic cell death.

2. Investigations and results

2.1. Tumour cell killing induced by Hypericum p. extracts

The tumour cell lines (K562, U937, LN229) and NHA were exposed to A (fresh) and B (dried) *Hypericum p.* extracts for 48 h in the absence of illumination and under light exposure of 7.5 J/cm^2. As the photosensitive agent, their constituent hypericin at a concentration of 1 μM was used. Dose- and light-dependent cell responses were established (Fig. 1 a–d). Comparison of LC_{50} values obtained (Table 1) showed that the viability of all cell lines tested were affected comparably under the test condition without exposure to light, a range from 1.89–3.15 mg/ml for extract A and from 2.65–4.10 mg/ml for extract B were determined. The corresponding concentrations of hypericin and hyperforin lay between 10.3–17.3 μM and 109.1–190.7 μM, respectively. Under illumination, the LC_{50} values decreased to 0.67–1.1 mg/ml for extract A and 0.93–1.75 mg/ml for extract B, which correspond to 3.7–7.4 μM hypericin. The light-dependent decreases in LC_{50} concentration in individual cell lines were between 2.8–3.4-fold for extract A and 2.3–2.9-fold for extract B. Hypericin at a concentration of 1 μM showed no toxicity in darkness, while under light condition about 20% of tumor cells (K562, U937, LN229) and NHA cells revealed a damaged cell membrane and took up trypan blue dye.

2.2. Hypericum p. extracts induce growth arrest on human malignant cells in the absence of illumination

Since recently there have been several reports concerning the growth inhibitory effect of quercetin and of light-activated hypericin, (both constituents of total *Hypericum p.* extracts) on the proliferation of various tumour cell lines [17, 18, 19, 21, 30, 31], we also investigated the growth inhibitory effect of whole *Hypericum p.* extracts and compared them with the effects of the individual component hypericin. Cell proliferation was measured inderectly by the cleavage of tetrazolium salt WST-1 to formazan by cellular enzymes, which correlates to the number of metabolically active cells in the culture. Furthermore, this assay gives an indication of the integrity of mitochondrial and also extramitochondrial NADH- and NADPH-dependent redox enzyme systems. Disturbances in these systems occur before nuclear and membrane changes in the cells are apparent. The dose-response curves for *Hypericum p.* extracts in the individual cell lines are shown in Fig. 2 (a–d). The cell lines of haematological origin were approximately equally sensitive (432.5–798.8 μg/ml for extract A, 2.4–4.3 μM for hypericin, 26.1–48.3 μM for hy-

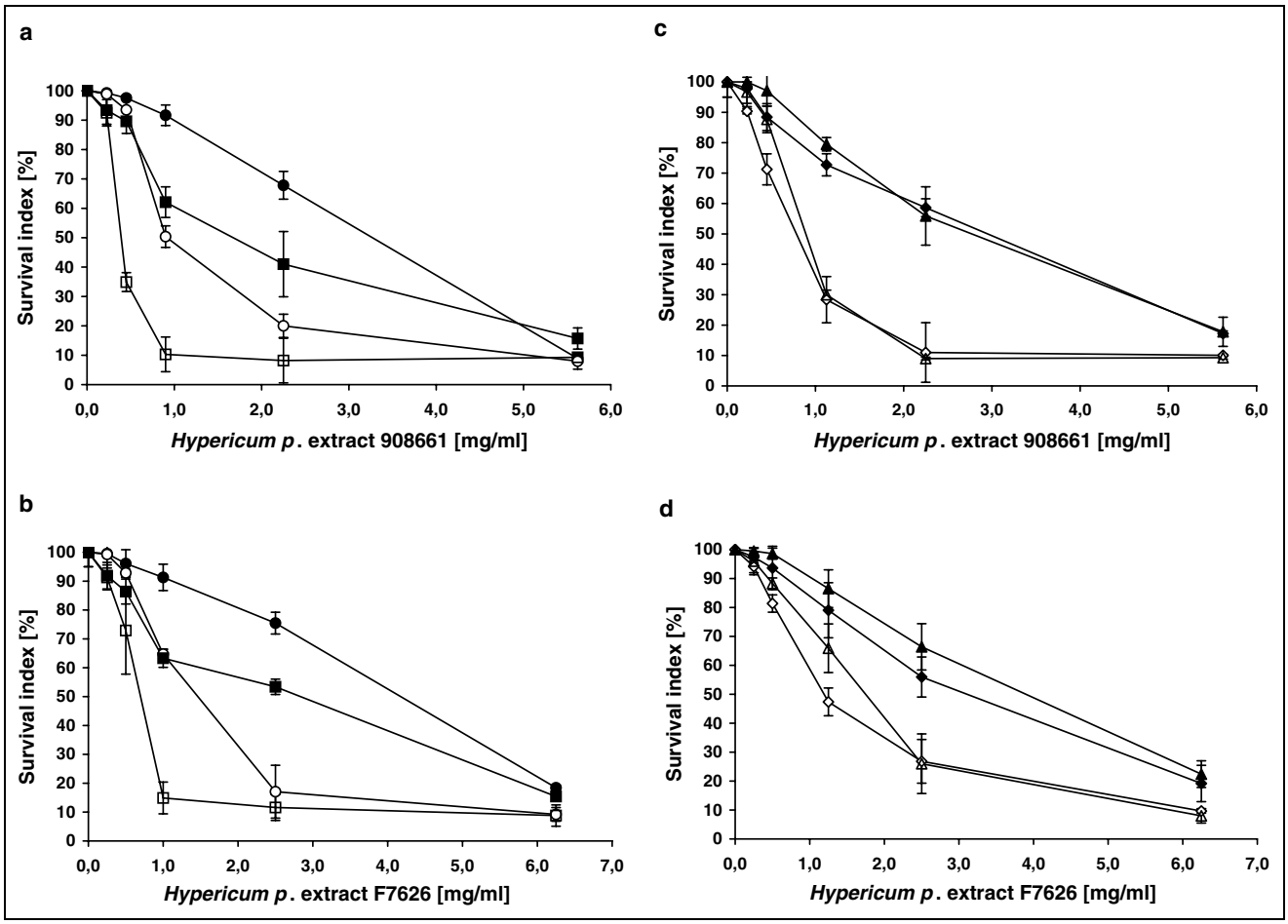

Fig. 1: Effect of *Hypericum p.* extracts on cell survival. Cells K562 (circles), U937 (squares), LN229 (diamonds) and NHA (triangles) were treated with increasing concentrations of *Hypericum p.* extract A (a, c) or B (b, d) in the absence of illumination (filled symbols) and after illumination of 7.5 J/cm^2 (open symbols) for 48 h. Data are expressed as mean percentages ± SD relative to untreated controls from three experiments performed in triplicate. Vehicle DMSO and ethanol (1 : 1) at concentrations of 0.2% did not affect the cell viability (data not shown)

Table 1: Lethal and cell growth inhibitory concentration 50% of fresh (A:908661) and dried (B:F7626) *Hypericum p.* extract

Cell line	Experiments without light exposure								Experiments by 7.5 J/cm^2 white light exposure							
	K 562	cells	U 937	cells	LN 229	cells	NHA	cells	K 562	cells	U 937	cells	LN 229	cells	NHA	cells
Extract [μg/ml]	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Mean LC_{50}	3150	4100	1890	2650	2970	3450	3130	4000	930	1410	670	930	922	1242	1103	1750
SD	132	131	124	304	243	224	323	396	62	84	123	215	105	122	105	233
p value		0.01		0.03		0.20		0.06		0.02		0.09		0.07		0.06
Mean GI_{50}	432	587	799	1030	1767	3030	2900	4033	282	309	505	505	582	840	1050	1067
SD	39	119	143	128	368	147	757	153	51	43	165	158	45	36	50	115
p value		0.03		0.03		0.02		0.10		0.31		1.00		0.01		0.84

* p values are expressed for differences of means between extract A and B
Means were established from three experiments performed in triplicates

perforin), whereas concentrations of up to 1.77 mg/ml (9.7 μM hypericin, 106.7 μM hyperforin) and 2.9 mg/ml (15.9 μM hypericin, 175.6 μM hyperforin) of extract A were required for half-maximal inhibition of cell growth in LN229 and NHA cells. The differences between K562, the most sensitive cells, were 4.1-fold for LN229 and 6.7-fold for NHA. The same tendencies in sensitivity and GI_{50} values for extract B were observed. However, there were significant differences ($p < 0.05$, two-tailed t test) between the growth inhibitory effects of fresh extract A and dried extract B in tumour cell lines K562, U937 and LN229 expressed in GI_{50} (Table 1). After light-activation of *Hypericum p.* extracts, the GI_{50} values were lowered being between 282.3–581.7 μg/ml (1.5–3.2 μM hypericin) for extract A in 3 tumor cell lines, K562, U937 and LN229. The half-maximal NHA cell growth inhibitory concentra-

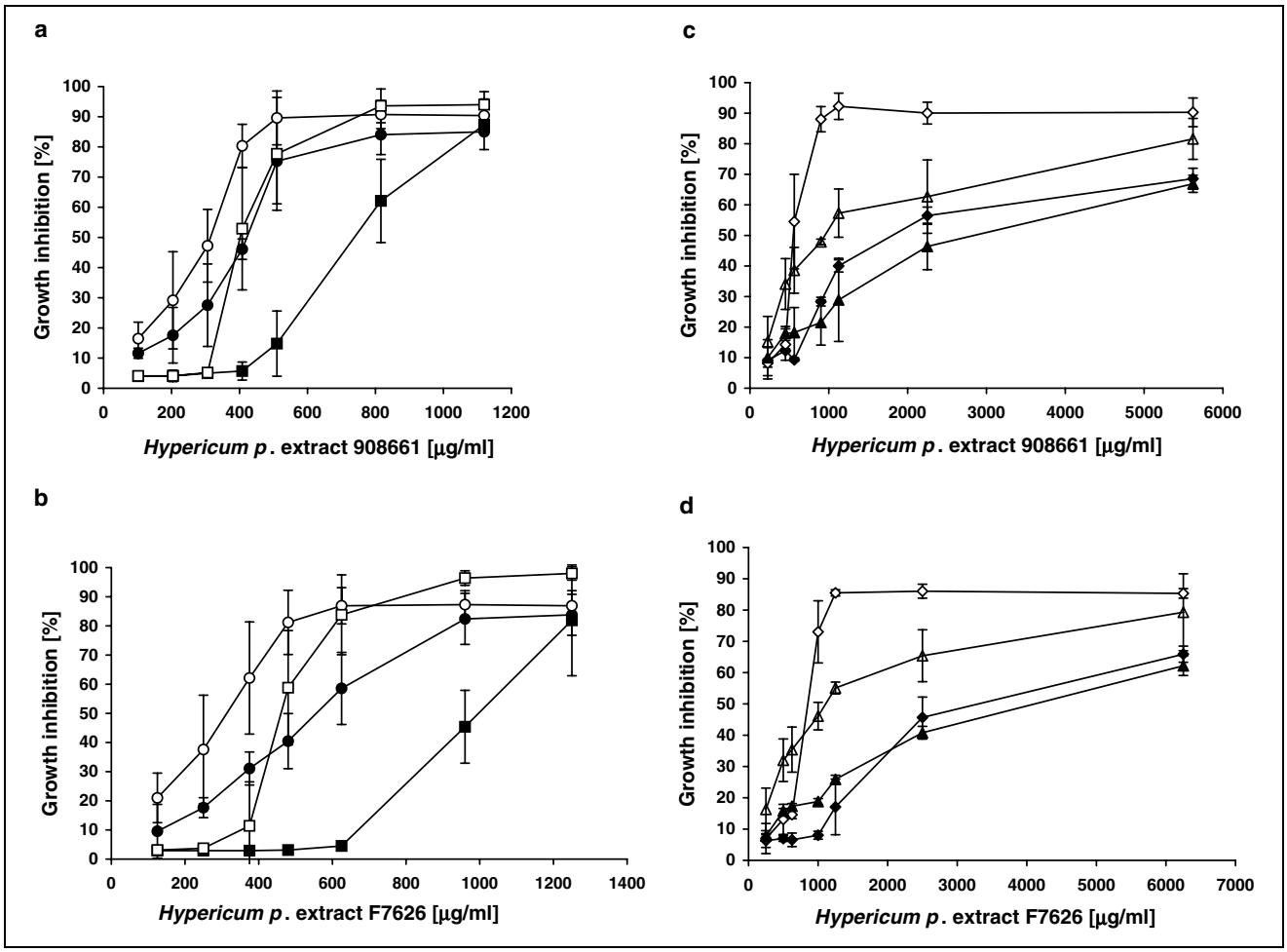

Fig. 2: Effect of *Hypericum p.* extracts with or without light exposure on cell growth. Cells K562 (circles), U937 (squares), LN229 (diamonds) and NHA (triangles) were incubated for 1 h with various concentrations of *Hypericum p.* extract in the dark and then subjected to 7.5 J/cm^2 light exposure (open symbols) or left in the dark (filled symbols). Untreated cells and cells incubated in the presence of vehicle were used as controls. In all experiments the values of vehicle-treated cells did not exceed the intra-experimental variance, which was less than 10%. Individual dose-response curves for each cell line from triplicates repeated at least three times with similar results are expressed as mean growth inhibition percentages ± SD

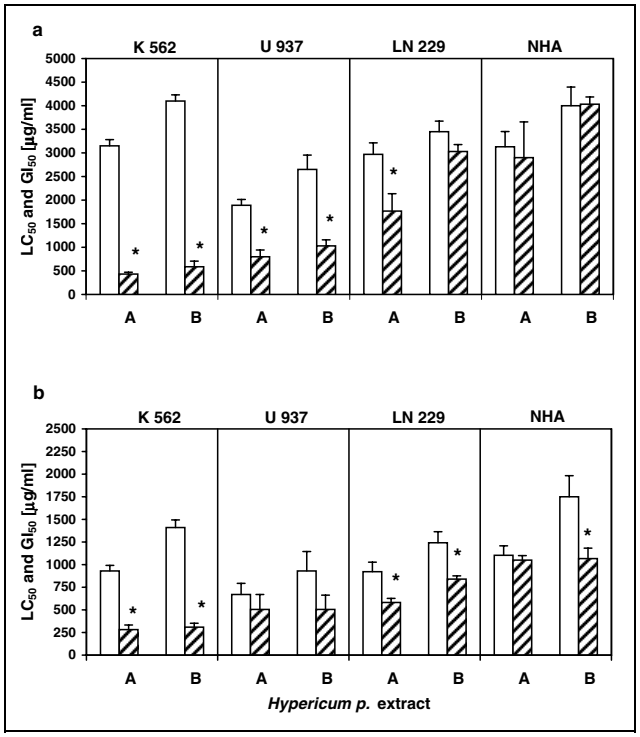

Fig. 3: Lethal concentration 50 (drug concentration causing 50% of cell death) and growth inhibitory concentration 50 (compound concentration which inhibited cell growth by 50%) of *Hypericum p.* extracts in the different cell lines. The LC_{50} values (open bars) and GI_{50} (hatched bars) of *Hypericum p.* extracts A and B were established by regression analysis fitted with Microsoft Excel software. The mean values + SD from at least three individual experiments carried out in the dark (a) and in 7.5 J/cm^2 light exposure (b) are presented. Statistical significances $p < 0.05$ between LC_{50} and GI_{50} are indicated *

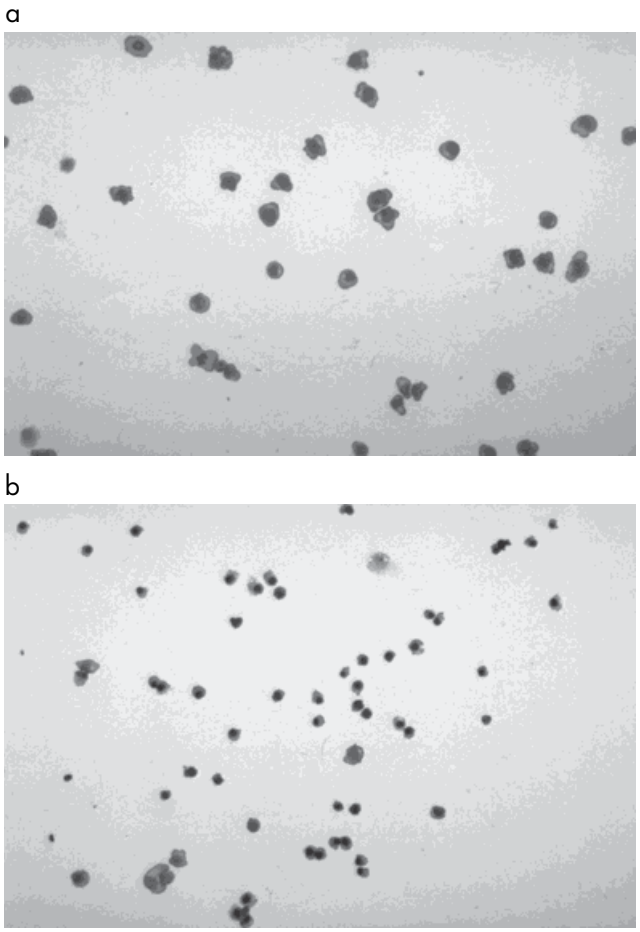

Fig. 4: Morphological changes of K562 cells after *Hypericum p.* extract treatment. Cells treated for 24 h with 0.9 mg/ml fresh plant *Hypericum p.* extract (b) in comparison to untreated controls in the dark (a) after Giemsa staining at 100 × magnification are shown

tion was 1.05 mg/ml (5.8 µM hypericin) for extract A which differed significantly ($p < 0.05$) from GI_{50} values for these three malignant cell lines. Under illumination, there were no significant differences between the effects of extracts A and B except in LN229 ($p < 0.01$). The lowered GI_{50} concentrations observed after light exposure differed significantly ($p < 0.05$) in all cell lines from the values obtained without illumination due to the phototoxic effect of hypericin. In Table 1, the mean LC_{50} and GI_{50} concentrations from experiments performed at least three times in individual cell lines are summarized. The comparison between the half-maximal lethal- and growth inhibitory-concentrations is shown in Fig. 3. The ratio LC_{50}/GI_{50} for *Hypericum p.* extracts in the sensitive leukemia cells (U937 and K562) lay between 2.4 and 7.3, whereas for brain astrocytes and LN 229 glioblastoma cells the range was narrow (1.0–1.7). The growth inhibitory effects of *Hypericum p.* extracts in LN229 and NHA cells are closely related to their toxicity. After illumination, these ratios were more homogenous being 1.0–1.8 in U937, LN229 and NHA. The greatest variations, i.e. 3.2 and 4.5-fold were detected in the most sensitive K562 cells for extracts A and B, respectively.

2.3. Morphological changes induced by Hypericum p. extracts

Cell were treated with *Hypericum p.* extracts for 24 h and then examined by light microscopy for morphologic changes typical of apoptosis. Compared with untreated cells, K562, U937, LN229 and NHA exhibited characteristic alterations such as shrinkage of total cell volume, reduction in nuclear size and chromatin condensation at the nuclear periphery. The apoptotic effect of *Hypericum p.* extract A on K562 cells is shown in Fig. 4. The morphological changes were confirmed by light scattering characteristics of apoptotic cells using flow cytometry. Representative dose and light dependent changes of the forwards (FSC) vs side (SSC) scatter of K562 cells are shown in the inset of Fig. 5.

2.4. Effect of Hypericum p. extract on the induction of apoptosis

The purpose of this study was to evaluate whether the cytotoxic and antiproliferative actions of *Hypericum p.* extracts on human malignant cells could be due to induction of apoptosis. We first quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V. Control cells, or cells treated with *Hypericum* extracts, (1 h in the dark) were exposed to light (7.5 J/cm^2) and incubated for a further 23 h. Cells were rinsed twice with PBS, stained with AnnexinV-FITC and analyzed by flow cytometry. Fig. 5A shows a representative experiment with K562 cells after treatment with *Hypericum p.* extract A in the absence of illumination. The apoptotic response of these cells to *Hypericum p.* extract A after light exposure is presented in Fig. 5B. In the dark, 5.1%, 15.1% and 35.5% of

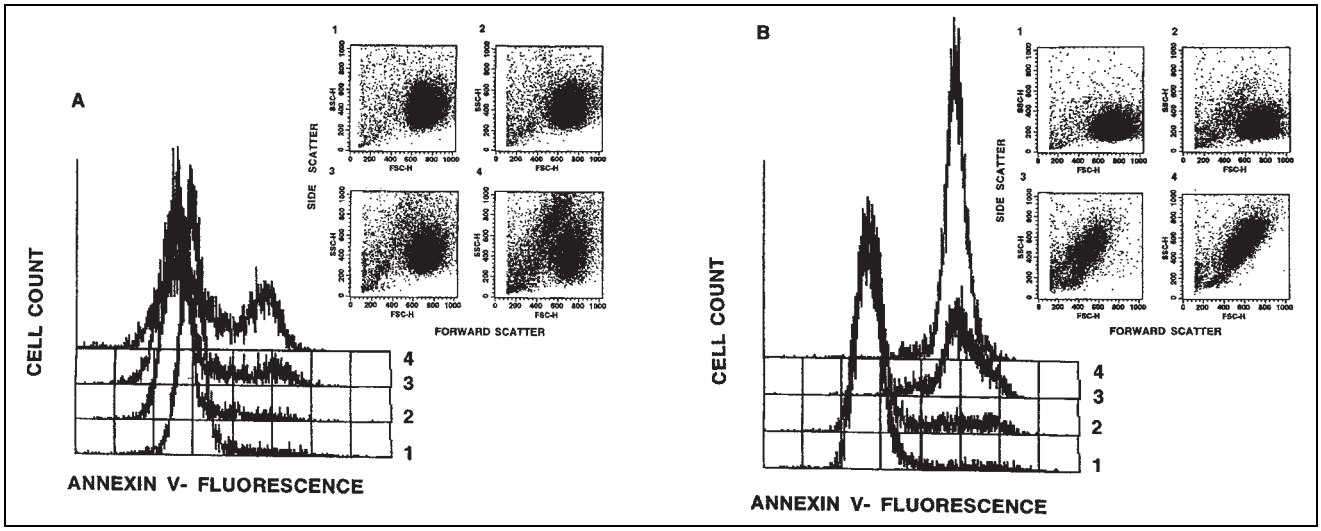

Fig. 5: Effect of *Hypericum p.* extract (A: 908661) with or without light exposure on apoptosis in K562 cells. Control cells (1) or cells treated with increasing concentrations (2: 0.45 mg/ml, 3: 0.90 mg/ml, 4: 1.12 mg/ml) of *Hypericum p.* extract (1 h in the dark) were exposed to 7.5 J/cm^2 light (B) or kept in the dark (A). After further 23 h incubation, the cells were stained with Annexin V-FITC and analyzed by flow cytometry. Annexin V-positive cells are presented as histograms numbers 2, 3, and 4. Results were compared with untreated controls (histogram 1). Parallel changes in the scatter-characteristic of cells are shown in the insets. Appropriate control experiments were performed to exclude the possibility that the solvents for the compounds, ethanol and DMSO, had effects on apoptosis at the final concentrations used

Annexin V-positive cells were found at doses of extract A of 0.45, 0.90 and 1.12 mg/ml respectively as compared to spontaneous apoptosis in 3.9% of control cells. Under illumination, marked increases of 14.1%, 97.9% and 98.9% in the number of apoptotic cells were detected at concentration of 0.45, 0.90 and 1.12 mg/ml respectively of extract A as compared to 5.1% in untreated controls. The parallel changes in light scatter FSC and SSC of the cells are shown in the insets of Fig. 5. Extract B had comparable apoptotic-inducing ability (data not shown). Monocytic leukemia cell line U937 was also sensitive to apoptotic cell death after 24 h of treatment with 0.45, 0.90 or

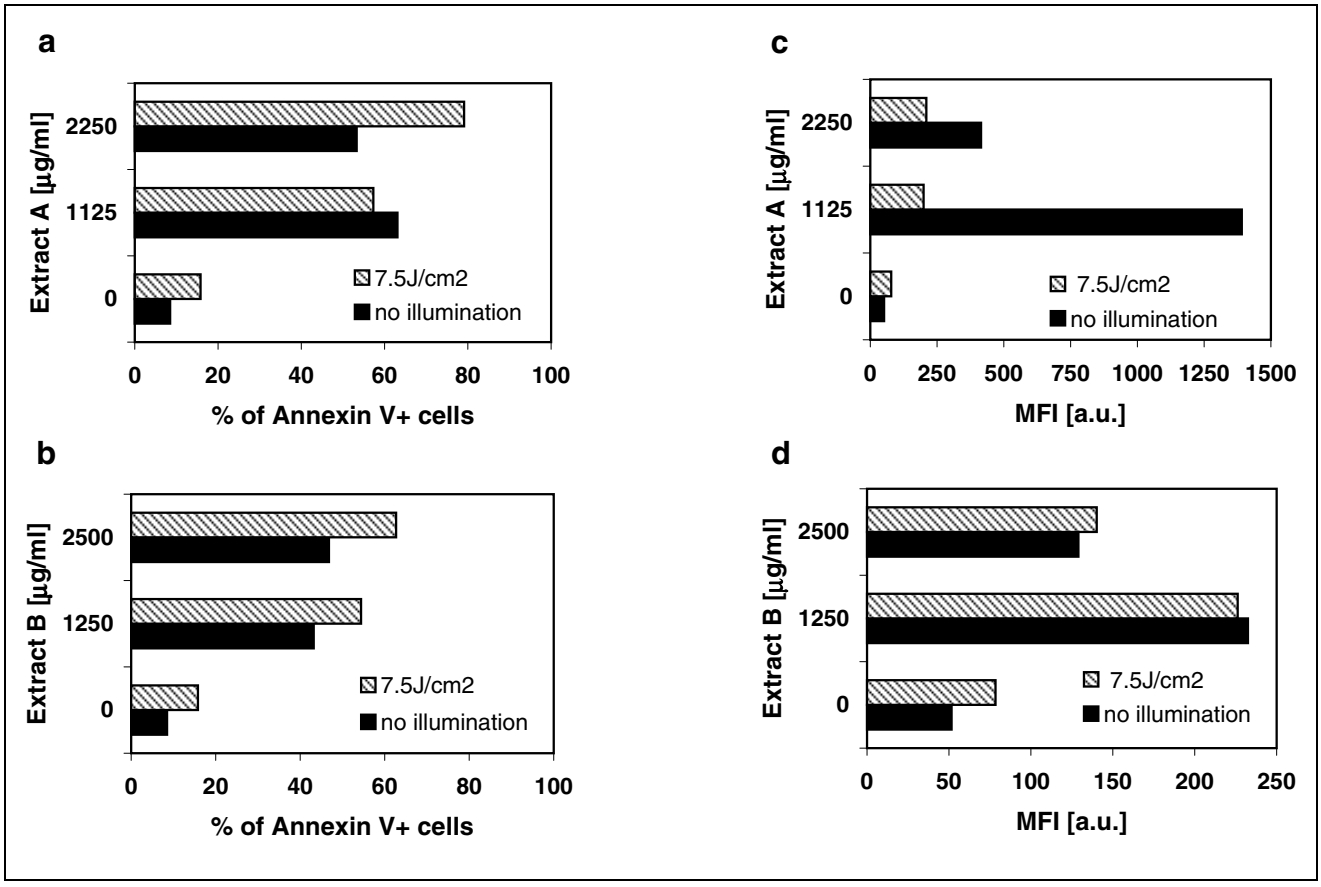

Fig. 6: Effect of *Hypericum p.* extracts on Annexin V adherence to glioblastoma cells. Annexin V-FITC binding to LN 229 cells treated with *Hypericum p.* extracts A (a, c) and B (b, d) in the dark (filled bars) and after 7.5 J/cm^2 light exposure (hatched bars) as analyzed by FACS in a representative experiment, expressed as percentage of Annexin V positive cells (a and b) and as average of fluorescence intensity (MFI) of Annexin V adherence to cells in arbitrary units (a.u.) (c and d). Experiments were performed twice with similar results

1.12 mg/ml of extract A or B which resulted in 35.7%, 52.1% and 68.2% of apoptotic cells, the spontaneous apoptotic rate of these cells being 25.7%. Light activation resulted in a massive increase in apoptotic cells from 35.4% (vehicle treated) to 65.3% (0.45 mg/ml A) and 93.7% (0.90 mg/ml A). Treatment of K562 and U937 cells with light-exposed extract in concentrations corresponding to 4.4–5 μM hypericin and 48.9–55.6 μM hyperforin that were about 1/3 of the LC_{50} showed more than 90% Annexin-positive cells. There were no significant differences between apoptotic induction (%) of extract A or B on either of the two leukemia cell lines and the variation in mean fluorescence intensity (MFI) did not exceed 2-fold values in any of the experiments. Glioma cell line LN229 and NHA were less responsive to apoptotic effects of *Hypericum p*. extracts. Apoptosis of the cells occurred in concentrations of the extract which approximated their LC_{50} values. The breakdown of asymmetrically distributed plasma membrane phosphatidylserine exposed on the outer layer of the cell membrane as assessed by Annexin V binding is presented in Fig. 6 and Fig. 7 for LN229 and NHA cells, respectively. The percentage of Annexin V positive cells for extracts A and B were comparable, but the intensity of Annexin-binding expressed as mean MFI in arbitrary units (a.u.) differed significantly in glioblastoma LN229 cells in experiments without light exposure. Further, there was a difference of ten on the logarithmic scale in MFI between LN229 and NHA cells at a concentration of 1.125 mg/ml of extract A. Elevations of MFI compared to untreated controls were 26.9-fold and 2.2-fold for LN229 and NHA, respectively, the background values being 51.6 and 46.3 (a.u.). The experiments were performed twice with similar results. There was no loss of plasma membrane integrity after 24 h treatment of cells with *Hypericum p*. extracts, with or withouth light exposure as detected by trypan blue dye. Light-exposed hypericin (5 μM) induced 70% Annexin V-positive K562 and U937 cells and about 40–50% LN229 and NHA cells showed Annexin V adherence.

3. Discussion

The present study was designed to investigate whether *Hypericum p*. extracts exert cytotoxicity towards malignant cells by inducing apoptosis and whether this effect is light-dependent as with hypericin. We investigated *Hypericum p.* extracts from fresh (A) and from dried (B) plants in parallel. The aqueous alcoholic extracts from *Hypericum p.* contain most of the secondary metabolites i.e. flavonoids, procyanidines, tanning agents, the naphthodianthrones pseudohypericin and hypericin, the phloroglucinols hyperforin and adhyperforin, xanthones, and essential oils all of which can exert various biological activities [6–8, 11, 17–20]. Due to the fact that the total amount of secondary metabolites in the extract depends on the harvest period [32] extraction and production procedure [33–35], both *Hypericum* p. extracts (A and B) were characterized by HPLC before using them in the biological test system. Concerning hypericins and flavonoids, both extracts exhibited comparable total amounts of these groups of constituents, whereby there were noteworthy differences in the amount of the phloroglucinols hyperforin and adhyperforin. The phloroglucinol content of fresh extract A exceeded that of dried plant extract B by 47%. Viability studies showed that, without exposure to light, the toxicity of the *Hypericum p.* extracts was low in all cell lines investigated as expressed by their LC_{50} values in mg/ml concentrations (Table 1, Fig. 1). Under light exposure, the half-lethal and growth inhibitory concentrations decreased as expected, due to the known phototoxicity of hypericin for a broad panel of malignant cells [14–18].

In addition, we compared the antiproliferative effect of both extracts and our results suggest (Table 1, Fig. 2) differences in sensitivity among the cells to growth inhibitory activity of extracts in the dark: (i) between leukemia cell lines K562, U937 and glioma LN229 and NHA cells. The growth inhibitory effects of *Hypericum p*. extracts after 48 h were more pronounced in the K562 and U937 cells. GI_{50} values 432 μg/ml (K562) and 587 μg/ml (U937) differed significantly ($p < 0.05$) from those of

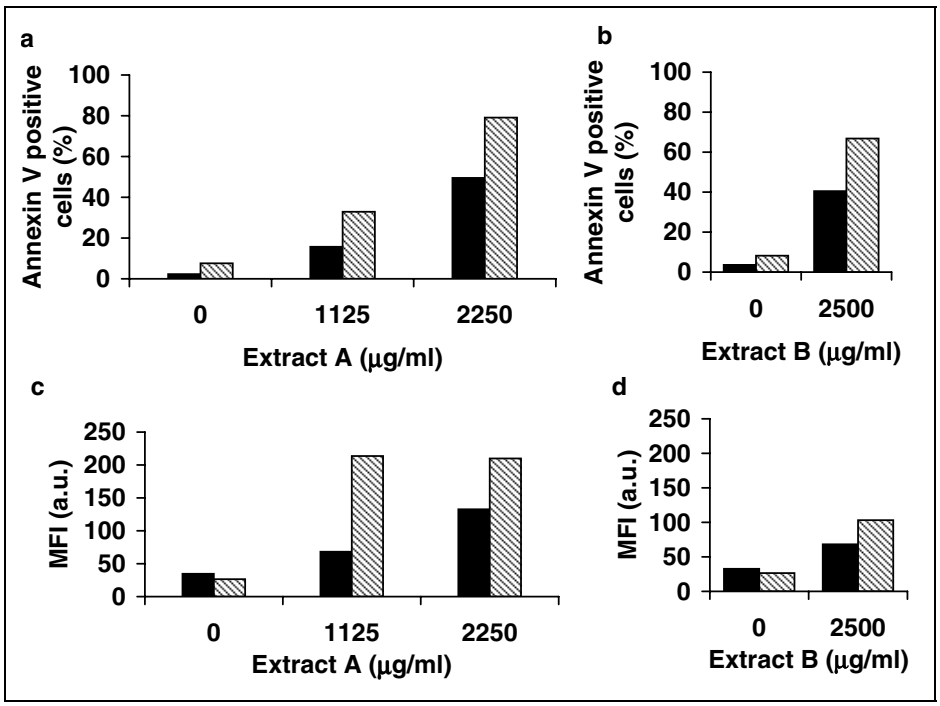

Fig. 7:
Phosphatidylserine exposure on normal human astrocytes after treatment with *Hypericum p*. extracts. Annexin V-FITC binding to NHA cells after 23 h postirradiation of extract A (a, c) and extract B (b, d) treated cells are presented as percentage of Annexin V positive cells (a, b) and as MFI (a.u.) of Annexin V (c, d). Filled bars represent experiments in the dark and hatched bars are data from illuminated (7.5 J/cm^2) experiments. A representative of two experiments with comparable results is shown

LN229 (1767 μg/ml) and NHA cells (2900 μg/ml). In the leukemia cell lines, there were significant differences between the LC_{50} and GI_{50} values of extract A (7.3-fold variation, $p < 0.001$ for K562 cells and 7.0-fold variation for U937 cells $p < 0.001$). However in less sensitive LN229 and NHA cells these concentrations lay within a narrow range, variations being only 1.68 and 1.08-fold (Table 1 and Fig. 3). The growth inhibitory effect in these cells were related to the toxicity of the extract. (ii) There were significant differences in the growth inhibitory effect between extracts A and B on three tumour cell lines ($p = 0.034$, K562; $p = 0.032$, U937 and $p = 0.024$, LN229). Because extract A contains a higher amount of hyperforin and adhyperforin than extract B, we hypothesize that phloroglucinols are involved in the growth inhibitory effect of *Hypericum p.* extracts in the dark. To further clarify this question, experiments were conducted and results will be reported in a separate publication.

Glioma cells LN229 were more sensitive to light-activated treatment with both extracts (A and B) compared to NHA cells ($p < 0.005$). These data suggest that tumor cells LN229 are more sensitive to light-activated toxicity of hypericin than NHA. The growth inhibitory effect of *Hypericum p.* extracts is potentiated by light exposure, but the illumination is not essential compared with hypericin, the toxicity of which is strong light-dependent. These data are in agreement with results reported by Bernd et al. [22] showing that higher concentrations of a methanolic *Hypericum p.* extract inhibited the growth of human keratinocytes even without light exposure.

In recent years, apoptosis has become an important issue in biomedical research. The life spans of both normal and cancer cells within a living system are considered to be substantially affected by the rate of apoptosis. Since apoptotic cell death differs from necrotic cell death and is regarded as an ideal physiological way of eliminating cells, we focused our attention on the potential apoptotic activity of our extracts. In the present study, several observations have been made concerning the apoptosis-inducing effect of total *Hypericum p.* extracts and the influence of light. Morphological changes inspected by light microscopy on Giemsa stained slides (Fig. 4) and the cell size characteristics (FCS, SSC) by flow cytometry (insets of Fig. 5) confirmed apoptotic cell death after 24 h treatment with *Hypericum p.* extracts. Apoptotic cells were also detected by the adherence of Annexin V to phosphatidylserine on cells membranes [36, 37]. Since, hypericin and consequently the extracts fluorescence in the red spectrum (~630 nm), we did not use propidium iodide dye for detection of necrotic cells but determined them by the trypan blue exclusion assay performed in parallel. A dose and light-dependent induction of apoptosis was observed in all cell lines examined. One representative example of this dependence is shown in Fig. 5 on K562 cells. After light exposure a massive increase of apoptotic cells was detected indicating that the light-toxicity of hypericin was effective.

The induction of apoptosis as expressed in % was comparable for both fresh and dried *Hypericum p.* extracts in the cell lines investigated. The leukemia K562 and U937 cells were equally high responsible to *Hypericum p.* extract exposure. The tumor cell line LN229 was more sensitive to apoptosis than NHA. About 50% of apoptotic cells were observed with 2.25–2.5 mg/ml of extracts (10.5–12.4 μM hypericin, 102.9–136.2 μM hyperforin) in NHA as compared to glioma cells LN229, where 50% apoptotic cells were reached with already half the concentration (1.25 mg/ml; 5.2–6.2 μM hypericin, 51.4–68.1 μM hyperforin) (Fig. 6 and 7). Interestingly, no increase in the % of apoptosis was observed by increasing the concentration above 1.25 mg/ml. These results were confirmed by the fluorescent values on the apoptotic cells. In fact, a decline in the MFI was detected, which suggest that, at higher concentrations, more cells died via necrosis. Remarkably, at a concentration of 1.25 mg/ml, the intensity of fluorescence of Annexin-V peaked at 235 a.u. with extract B compared to 1391 a.u. with extract A (Fig. 6). The MFI of Annexin V adherence to LN229 cells was higher than for NHA, suggesting a difference in the concentration inducing apoptosis between normal astrocytes and the glioma cells.

The present study is, to our knowledge, the first demonstration that *Hypericum p.* extract triggers apoptosis in various tumor cells including K562, U937 and LN229 and characterizes it as a potentially effective apoptosis-inducing agent. The results were validated by confirming the induction of apoptosis by hypericin as previously described by others [16–18]. The high sensitivity of leukemic cells to extract-induced apoptosis described in the present study could have a possible relevance for a photodynamic therapy in leukemia as has also been reported for the extract's metabolite hypericin [16]. We chose to investigate the glioma cells, since the prognosis of glioma patients is very poor [38]. This fact stimulated a search for new agents, which can modulate apoptosis and can be applied continuously without major toxicity and side effects. Hypericin, the main photosensitizing constituent of *Hypericum p.* extract has been proposed as a natural photosensitizer for photodynamic cancer therapy [39–41]. An *in vivo* antitumor effect of photoactivated hypericin has also been demonstrated [42, 43]. In addition, hypericin inhibited the growth of glioma in a dose- and light-related manner in tissue culture and induced apoptosis [17, 18, 44, 45]. Apoptosis-inducing effect of hypericin on a panel of seven human glioma cell lines provided evidence that the effect of hypericin is independent of two genes associated with apoptosis bcl-2 and p 53 [18] and binds to and triggers the mitochondria of glioma cells [17].

In conclusion, we could show that *Hypericum p.* extracts have a low toxicity and induce apoptosis in human glioma cells even without light exposure. Comparison between fresh and dried extracts showed that the effect of fresh plant extract was more pronounced than that of dried extract in the dark. Cancer patients often use this extract as an antidepressant. The *Hypericum p.* extract, as many other herbal products, is a multicomponent mixture with additional pharmacologically active substances, including antibacterial, antiretroviral [46–47], antioxidative [48] and antiinflammatory [23, 49] activity as well as the purified constituents such hypericin, hyperforin, and flavonoids responsible for the documented effects [47, 50–52]. However, the question arises whether the use of total extract will be superior to pure constituents because of the possible synergism between them. Evidence that the effect of the total extract could differ from its single constituents was recently reported by Vandenbogaerde et al. [53]. The authours could show that the total extract of *Hypericum p.* increases locomotor activity in the open field and exerts anxiolytic activity in the light-dark test, whereas the pure components protohypericin, hypericin and pseudohypericin were not active. On the other hand, only hypericin was characterized as an inhibitor of NF-κB [54]. *Hypericum p.* extract and hyperforin could not inhibit the activa-

tion of NF-κB on HeLa cells. Recently, a synergistic effect between *Hypericum p*. extract and another photosensitizer δ-aminolaevulinic acid was described [55]. This observation could probably open a new perspective for the use of *Hypericum p*. extract in the photodynamic diagnosis of cancer. *Hypericum p*. extract is well tolerated by patients even at high doses [56] and has no mutagenic potential [57]. Further *in vitro* and *in vivo* studies are required to determine the potential of *Hypericum p*. extract, which contains a number of pharmacologically active constituents, all of which may potentially participate in its apoptosis inducing effect.

4. Experimental

4.1. Hypericum p. extracts

The ethanolic *Hypericum p.* extracts (35% w/w) ID: 908661 (fresh plant, A) and F7626 (dried plant, B) with a drug to extract ratio of 1.3–1.5 : 1 were supplied from Bioforce AG (Roggwil, Switzerland). The concentrations of three main groups of extract components quantified by HPLC are summarized in Table 2. Naphthodianthrones are expressed as hypericin (MW: 504.4), phloroglucinols as hyperforin (MW: 536.8) and flavonoids as rutin trihydrate (MW: 664.5). Hypericin was from Alexis Corporation, Lausen, Switzerland. *Hypericum p*. extracts and hypericin were dissolved in the mixture of DMSO and ethylalcohol (1:1). Hypericin was stored at a concentration of 5 mg/ml in aliquots at −20 °C. The final concentrations of DMSO and ethylalcohol in cells were 0.2% and did not affect viability, cell growth and apoptosis.

4.2. Cell lines

K562 human chronic myeloid leukemia-(CCL 243) and U937 human histiocytic lymphoma-(CRL 1593), were obtained from the American Type Culture Collection (Rockville, MD, USA). Normal human astrocytes (NHA-5989-1) from the first passage of a single donor (Clonetics, BioWhittaker, Verviers, Belgium) were used. Human malignant glioma cells LN229 characterized by [58] were provided by Dr. N. de Tribollet (Lausanne, Switzerland). Cell culture media RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), phosphate buffered salt solution (PBS), media supplements fetal calf serum (FCS), L-glutamine, penicillin-streptomycin (PenStrep) were purchased from Sigma (Buchs, Switzerland).

4.3. Cell cultures and treatments

Cell cultures were maintained at 37 °C, in a 5% CO_2/95% air humidified incubator. Propagation medium for K562 and U937 cells was RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 1% PenStrep. LN229 were cultured in DMEM containing 10% FCS 2 mM L-glutamine and 1% PenStrep. The NHA were grown in Astrocytes Basal Medium (ABM™) with following supplements 5% FCS, 2 μg hEGF (human recombinant Epidermal Growth Factor), 2 mg insulin, 2.5 μg progesterone, 5 mg transferrin, 5 mg gentamycin, 5 μg amphotericin. ABM™ with all supplements listed above (AGM™ BulletKit) were obtained from BioWhittaker (Verviers, Belgium). For experiments, cells were harvested during the exponential growth phase (K562, U937) or were collected from subconfluent monolayers (LN229, NHA) with trypsin/EDTA. Cell viability was >95% before each experiment as estimated by trypan blue exclusion. The test medium for all experiments was 1640 RPMI or DMEM supplemented with 2% FCS. Cytotoxicity of individual compounds was determined by exposure of seeded cells 5000/100 μl to various concentrations for 1 h in the dark prior to light exposure. In parallel, wells were treated without exposure to light. After 48 h, viability and cell growth inhibition were estimated.

Table 2: HPLC analysis of *Hypericum p.* extracts

Charge number Ingredients	908661 (A) (mg/100 g)	F7626 (B) (mg/100 g)
Pseudohypericin	235.6	177.1
Hypericin	41.8	35.9
Naphthodianthrones[a]	277.4	213.0
Hyperforin	2772.0	1901.0
Adhyperforin	481.1	314.0
Phloroglucinols[b]	3253.1	2215.0
Flavonoids[c]	5294.0	5349.0

a – expressed as hypericin (MW: 504.4), b – as hyperforin (MW: 536.8) and c – as rutin trihydrate (MW: 664.5)

4.4. Light exposure

Cultured cells were illuminated as described [17]. Briefly, cells in microtiter plates were placed 20 cm under a 75 W tungsteam lamp for 10 min. The light dose was 7.5 J/cm^2. To reduce other sources of illumination the samples were protected from light with aluminium sheets. During irradiation, the temperature never exceeded 30 °C, which did not influence the cell viability.

4.5. Viability-Trypan blue stain

Trypan blue is a dye that enables easy identification of dead cells. Dead cells with damaged cell membrane take up the dye and appear blue; by contrast, living cells exclude it and appear colorless. Cell suspensions of detached and adherent cells harvested by centrifugation were stained with trypan blue solution (0.2% w/v in PBS) for 5 min and counted on a hemacytometer. The effect of test compounds on cell survival were determined as percentage of control cells grown without test substances. Cell viability was calculated using equation:

$$\text{Survival index } [\%] = \frac{\text{number of unstained cells}}{\text{Total cell count (stained + unstained)}} \times 100\% \quad (1)$$

The lethal concentration LC_{50} was defined as concentration which causes 50% cell death and was determined from three independent experiments.

4.6. Cell growth inhibition

Cell proliferation was indirectly measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) to formazan by mitochondrial dehydrogenases in viable cells [59]. After incubation for 44 h, 10 μl of WST-1 reagents were added to wells and incubated for a further 4 h in humidified atmosphere. The absorbance of the samples against a background control as blank using a microtiter plate (ELISA) reader at 450 nm wavelenght was measured. The reference wavelenght was 650 nm. Experiments in triplicate repeated three times were evaluated. Cell growth inhibition (%) was calculated from the formula:

$$GI = \left[1 - \frac{\text{absorbance of treated cells}}{\text{untreated control}}\right] \times 100 \quad (2)$$

The concentration which inhibited the cell growth by 50% was defined as GI_{50}.

4.7. Morphological investigations

The morphological changes consistent with apoptosis (cell shrinkage, chromatin clumping, nuclear fragmentation) were inspected by oil-immersion light microscopy of Giemsa stained cytospin slides as previously described [37].

4.8. Annexin V-FITC labelling

Apoptosis was detected and quantified in cells (5×10^4) by staining with the anticoagulant Annexin V conjugated with FITC from (Roche Diagnostics, Rotkreuz, Switzerland) as described [36, 37]. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine, which is exposed on the outer plasma membrane and is a hallmark for early apoptosis. Cultured cells were stained with FITC-conjugated AnnexinV in binding solution for 15 min at room temperature in the dark and analyzed on FACScalibur (B-D, Basel, Switzerland) equipped with the CellQuest program. Green fluorescence histograms from 10^4 cells were compared to control experiments. Each histogram is representative of two independent preparations. Plasma membrane permeability was estimated by trypan blue exclusion.

4.9. Statistical analysis

LC_{50} and GI_{50} values were obtained by linear or exponential regression analysis of data using the Microsoft Excel software. Statistical comparisons between means were performed by two-tailed Student's t-test. $P < 0.05$ was considered as statistically significant. The comparison of histograms were statistically evaluated using the Kolmogorov-Smirnov (K-S) two samples test for overlaid histograms.

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