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Comparison of the growth-inhibitory effect of *Hypericum perforatum* L. extracts, differing in the concentration of phloroglucinols and flavonoids, on leukaemia cells

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Abstract

In this study we compared, simultaneously, the growth-inhibitory effect of *Hypericum perforatum* L. extracts, containing various amounts of hyperforin (A 3.25%; B 2.21%; C 0.21% w/w) and flavonoids (A and B 5.3%; C 10% w/w), but closely same amounts of naphthodiantrones (0.3%) on two leukaemic cell lines K562 and U937 in the WST-1 assay. The GI50 (concentration of extracts which caused 50% of cell growth inhibition) for *H. perforatum* extracts analysed and characterized by HPLC for their biologically active constituents was 248.3–621.3 μg mL^{-1} in K562 and 378.2–911.7 μg mL^{-1} in U937 cells. The corresponding values of the three main groups were 1.6–3.9 μM naphthodianthrones, 1.0–40.7 μM phloroglucinols and 30.5–68.5 μM flavonoids. The results of this study supported the hypothesis that, apart from hyperforin and flavonoids, other components of the extract could be involved in its growth-inhibitory effect that it exerts without light activation.

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Introduction

Crude extracts of Hypericum perforatum L. (Clusiaceae) contain a wide range of compounds with pharmacological properties, such as naphthodianthrones, phloroglucinols, xanthones, proanthocyanidines, biflavones, flavonol derivates, phenylpropanes, essential oils and amino acids (Bombardelli & Morazzoni 1995). Hypericin, a component of H. perforatum extracts, exhibits light-dependent phototoxicity on the growth of various human malignant cells (Miccoli et al 1998; Lavie et al 1999) and there are also numerous studies on the antiproliferative effects of flavonoids (quercetin) in malignant cells (Uddin & Choudhry 1995; Kang & Liang 1997). The acylphloro-glucinol derivate hyperforin is the major non-nitrogenous secondary metabolite of H. perforatum. Apart from its characterized antibacterial properties (Gurevich et al 1971), it was found that hyperforin exerted inhibitory effects on human epidermal cells and on the proliferation of phytohaemagglutinin-stimulated peripheral blood mononuclear cells. In addition, it inhibited the growth of autologous MT-450 breast carcinoma in Wistar rats in-vivo (Schempp et al 2000, 2002). Recently, was reported that an H. perforatum extract exerted growth-inhibitory activity on leukaemic (K562, U937) cells, human glioblastona LN229 and normal brain astrocytes (Hostanska et al 2002).

The aim of this study was to investigate this activity in relation to the quantity of the metabolite hyperforin in extracts. Light-dependence was examined under exposure to 7.5 J cm^{-2} white light using hypericin as a phototoxic control. This paper describes the cell growth-inhibitory data of alcoholic hypericum extracts, which suggested that hyperforin and flavonoids are not the only active antiproliferative components of the extract but that other constituents of extract might modulate its efficacy.

Materials and Methods

Reagents

Hypericin ($C_{30}H_{16}O_{8}$,), quercetin ($C_{15}H_{10}O_7.2H_2O$), rutin ($C_{27}H_{30}O_{16}.3H_2O$) and etoposide were from Alexis Corporation, Lausen, Switzerland. Hyperforin ($C_{35}H_{52}O_4$) was isolated and purchased from Natural Product Department of Dr W. Schwabe GmbH & Co. (Karlsruhe, Germany) as previously described by Erdelmeier (1998). The isolated hyperforin fraction yielded about 18% adhyperforin, identified as a homologue of hyperforin (Maisenbacher & Kovar 1992) and characterized by spectroscopic methods (Rücker et al 1995). Reagents were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C in samples exclusive of hyperforin, which was stored at −80 °C. Acetonitrile, methanol, tetrahydrofuran and 85% phosphoric acid from Sigma (Buchs, Switzerland) and Milli-Qplus purified water were used as solvents for chromathography.

Cell proliferation reagent WST-1 was purchased from Roche Diagnostica (Rotkreuz, Switzerland).

Extracts

The H. perforatum extracts (A-908661; B-F7626; C-813400) were supplied and analysed by Research Department of Bioforce AG (Roggwil, Switzerland). Only A was a commercially produced extract; B and C were experimental extracts. H. perforatum extract A was prepared by ethanol–water maceration of fresh flowering tops (15–20 cm), extract B was obtained by maceration of flowering tops dried in the dark and extract C by aqueous–ethanolic percolation of the light-dried whole aerial parts of the plants (including leaves and stems) followed by processing to dry extract. The drug–extract ratio was 1.3–1.7:1. Extracts A and B were derived from the same harvested herb mixture, equally processed, differing only in the kind of herb material macerated. Extract A was prepared from fresh herb mixture and B from one dried at 40 °C in the dark, and were stored in the dark during extraction. Further, the macerates A and B were concentrated under vacuum fluid extraction to viscid fluid. The extracts were adjusted to a final ethanol concentration of about 30%. However, extract C, after adjusting the concentration of hypericin to 0.3%, was processed to brown powder by spray drying.

High-performance liquid chromatography (HPLC) conditions

Aqueous alcoholic extracts of H. perforatum were analysed by HPLC coupled simultaneously to UV diode array detector (Brolis et al 1998) and the individual constituents of the three main groups (naphthodianthrones, phloroglucinols, flavonoids) were quantified using external reference standards. Analyses of extracts were performed on reversed-phase columns (RP-18). The analyses were for a 20–40 min period at a flow rate of 0.8 mL min^{-1}. The injected volume of the samples was 20 μL. Chromatographic separation of flavonol glycosides (rutin, hyperosid, isoquercitrin, quercitrin), flavonol aglycone (quercetin), biflavones (I3,II8-biapigenin, I3′,II8-biapigenin) and naphthodianthrones (hypericin, pseudohypericin) was carried out using a gradient consisting of an acidic phosphate buffer and an eluent mixture of acetonitrile, methanol and tetrahydrofurane buffered with phosphoric acid. The wavelengths of the diode array detector were set at 360 nm (flavonols) and 590 nm (naphthodianthrones). The same concentration (0.8 mg mL^{-1}) of the extracts A, B and C was used for the flavonoids and naphthodianthrones quantification. The phloroglucinols were extracted with ethanol under exclusion of light and detected at 270 nm. Separation was carried out using solvents (A, acetonitrile; B, acidic phosphate buffer). The injected concentrations were 16 μg (extract A and B) and 132 μg (extract C) by the analysis of phloroglucinols content. Calibration curves were obtained by employing external reference standards: hypericin for naphthodianthrones, rutin for flavonoids and an external working standard for phloroglucinols (hyperforin is not commercially available). Integrated peak areas were plotted against the corresponding amount of the injected standards. The chromatographic data were recorded and processed by HP Chemstation and PE Turbochrom software.

Culture conditions and cell growth assay

To determine the cytotoxicity of the extracts, human leukaemia K562 and U937 cells from the American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin–streptomycin (Sigma), in a 5% CO_2, 95% air fully humidified atmosphere at 37 °C. The assay medium was 1640 RPMI supplemented with 2% FCS. 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 (Ishiyama et al 1996). Cytotoxicity of extracts, hypericin, hyperforin and flavonoids (rutin, quercetin) were determined simultaneously using WST-1 agent after exposure of 5×10^3 cells to various concentrations of the test substances in the dark or after the 7.5 J cm^{-2} light irradiation (20 cm distance from 75 W tungsten lamp over 10 min) for 48 h (Miccoli et al 1998). The absorbance of the samples against a background control as blank using a microtitre plate (ELISA) reader at 450 nm wavelength was measured. The reference wavelength was 650 nm. Six independent sets of experiments performed in triplicate were evaluated. H. perforatum extracts were dissolved in a mixture of DMSO and ethanol (1:1). Controls with the solvent DMSO and ethanol below 0.2% were tested in parallel. The growth inhibition rate was calculated as percentage of untreated controls and did not affect the cell growth. Extracts were compared using the GI50 value, the

concentration of individual compounds reducing cell growth by 50%. GI50 values were obtained from each dose–response curve by linear regression analysis of data using the Microsoft Excel software.

Statistical analysis

The exerted growth-inhibitory effect expressed as GI50 of three H. perforatum extracts in the dark and under 7.5 J cm^{-2} white light were compared using a one-way analysis of variance followed by paired comparisons of the concentration values GI50 with Newman–Keuls post-hoc test. Six independent experiments performed in triplicates were analysed for the differences between individual extracts and treatments. The type I error rate was set at 0.05 for all analyses. Probability values equal to or less than 0.05 were considered to be statistically significant.

Results and Discussion

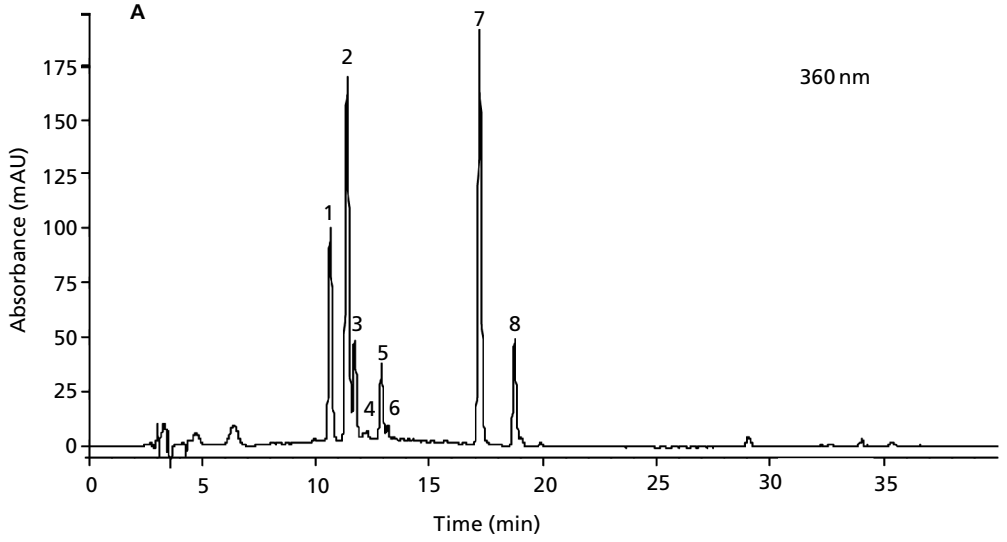
HPLC analysis of the individual compounds of *H. perforatum* extracts

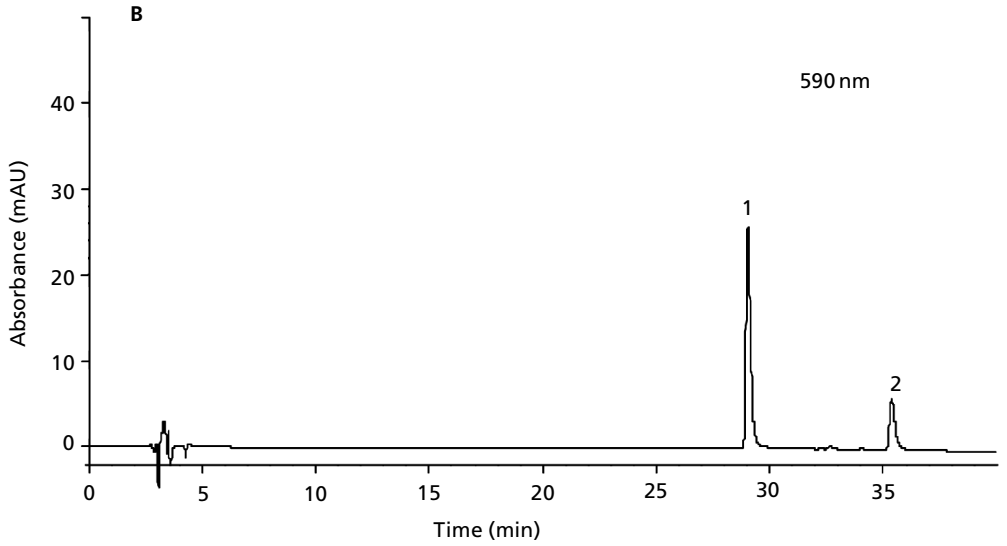
Before testing the extracts in the biological system, they were analysed by HPLC and the individual flavonoids, naphthodianthrones and phloroglucinols were quantified. The results are shown in Table 1 and representative chromatograms of the three main groups of H. perforatum extract A and C are presented in Figure 1. Concerning the amount of naphthodianthrones, there was only a small alteration (0.21–0.32% w/w). We chose the three different H. perforatum extracts mainly on the basis of their differences in phloroglucinol content (A 3.25%; B 2.21%; C 0.21% w/w). The negligible concentration of hyperforin in extract C was due to the processing, in which chlorophyll was also precipitated with hyperforin. In addition, the flavonoid content in extract C was about 10% compared with extracts A and B, which contained 5%. Analyses of the individual flavonoids showed that the most abundant flavonoids in extracts A and B were hyperoside/isoquercitrin (1%) and quercetin (2.8–3.1%). In extract C hyperoside/isoquercitrin (4.2%) and rutin (3.4%) dominated. However, extracts A (2.8%) and B (3.1%) were richer in quercetin than extract C (0.72%). The high amount of quercetin in both extract A and B could be related to the extraction procedure by which flavonol glycosides degrade to quercetin. There were differences in the contents of, for us, unknown (x, y, u) flavonoids between the extracts A (0.3%) and B (0.2%) and extract C (1.1%). According to newly described phenolic compounds of the H. perforatum extract (Jürgenliemk & Nahrstedt 2002), they could be possibly identified as the flavonoids miquelianin, astilbin and quercetin-3-O-(2″-O-acetyl)-β-D-galactoside. We examined the H. perforatum extracts prepared from the aerial part of herbs, because the individual chemical constituents investigated in this study accumulate predominantly in the flowers, stems and leaves. Hyperforin occurs only in the reproductive parts of the plant (flowers, ripe fruits). The amount of constituents in the extract depends on the quantity of each part in the processing mixture, harvest time and extraction procedure (Meier 1999; Tekelova et al 2000). Recently, the highest percentages of hyperforin and its derivates were found in extracts obtained from fresh samples with significantly lower amounts of these derivates being detected in air-dried samples. The content of hyperforin in samples obtained by light-extraction was diminished to a negligible content (Bergonzi et al 2001). Our HPLC analysis of hypericum extracts produced by maceration of fresh and dried herbs was in accordance with the aforementioned findings. The amount of hyperforin in extract B (derived from dried flowering tops) was reduced

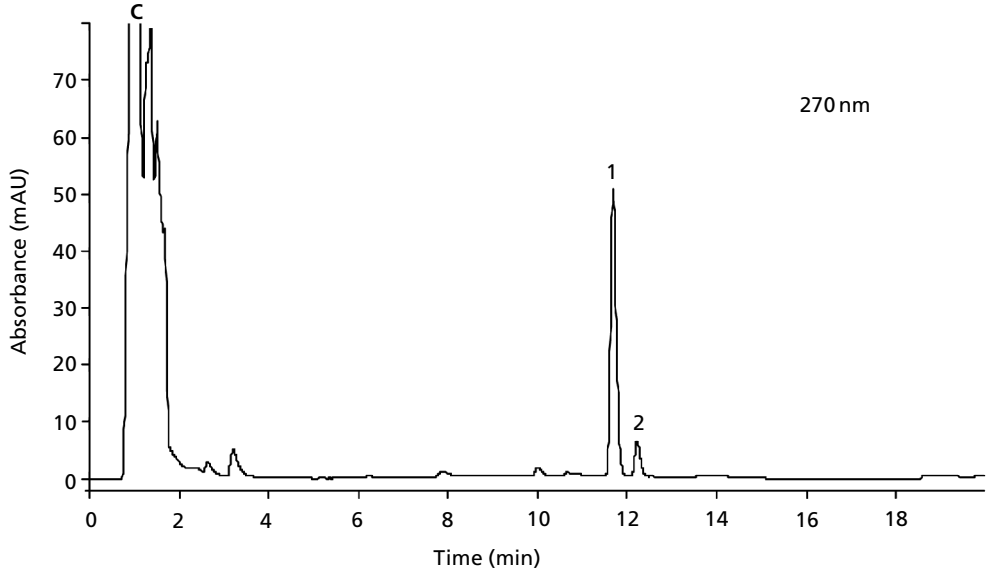
Table 1 HPLC analysis of Hypericum perforatum L. extracts.

Constituents	A-908661 (mg/100 g)	B-F7626 (mg/100 g)	C-813400 (mg/100 g)
Rutin	585.0	655.0	3357.0
Hyperoside/isoquercitrin	1080.0	1003.0	4228.0
Flavonoid x	210.0	137.0	651.0
Flavonoid y	110.0	30.0	315.0
Quercitrin	204.0	141.0	447.0
Flavonoid u	0.0	49.0	154.0
Quercetin	2794.0	3146.0	715.0
Biapigenin	310.0	187.0	219.0
Flavonoids	5294.0	5349.0	10085.0
Pseudohypericin	235.6	177.1	270.1
Hypericin	41.8	35.9	47.5
Naphthodianthrones	277.4	213.0	317.5
Hyperforin	2772.0	1901.0	181.0
Adhyperforin	481.1	314.0	29.5
Phloroglucinols	3253.1	2215.0	210.4

Naphthodianthrones are expressed as hypericin (MW 504.4), phloroglucinols as hyperforin (MW 536.8) and flavonoids as rutin $3H_2O$ (MW 664.5).


A
360 nm
Absorbance (mAU)
Time (min)
1
2
3
4
5
6
7
8


B
590 nm
Absorbance (mAU)
Time (min)
1
2


C
270 nm
Absorbance (mAU)
Time (min)
1
2

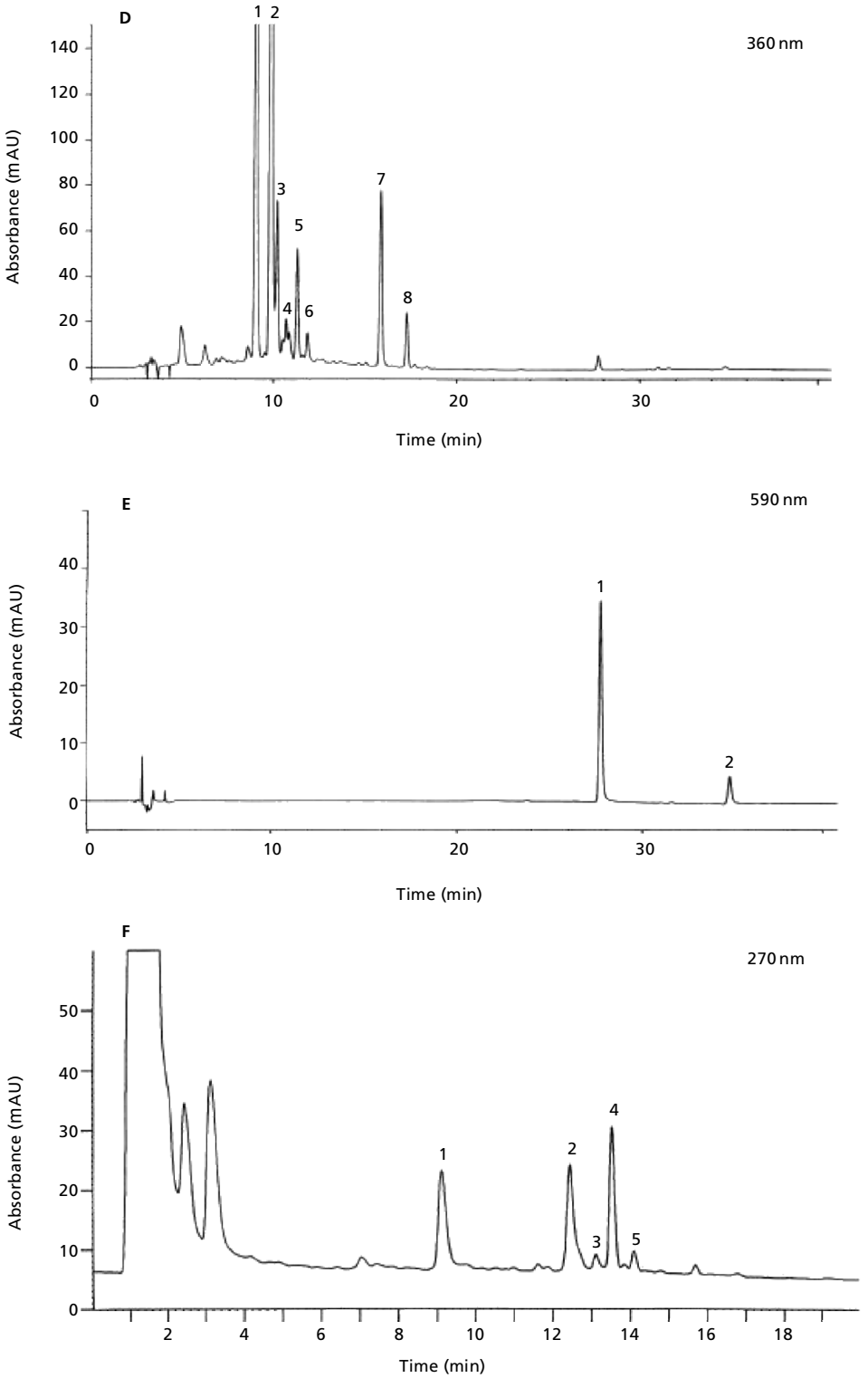


Figure 1 Chromatograms of the H. perforatum extracts A-908661 (A–C) and C-813400 (D–E) after separation of naphthodianthrones, phloroglucinols and flavonoids on RP-18 column. In A and D the HPLC profiles of the flavonoid compounds of extracts at 360 nm are shown. Peaks are assigned as 1 = rutin, 2 = hyperoside/isoquercitrin, 3 = x, 4 = y, 5 = quercitrin, 6 = u, 7 = quercetin, 8 = I 3, II 8-biapigenin (x, y and u are components not known to us). HPLC profiles of the naphthodianthranoid compounds of H. perforatum extracts detected at 590 nm with peaks 1 = pseudohypericin and 2 = hypericin are shown in B and E. HPLC profiles of phloroglucinols at 270 nm wavelength are presented in C and F. Peaks are assigned as 1 and 4 = hyperforin, 2 and 5 = adhyperforin (C and F, respectively) and 1, 2, 3 for the components not known to us (F). The injected concentrations of the extracts A, B and C for the phloroglucinols analysis were 16 μg (extracts A, B) and 132 μg (extract C).

by 33% compared with its content in extract A (from fresh flowering tops) and in extract C (from light-dried herbs) its content was only 0.2%. There were differences in the chromatograms of phloroglucinols between extract C compared with extracts A and B. Phloroglucinols from extracts A or B included only hyperforin and adhyperforin (peaks 1 and 2; Figure 1C). However, on the chromatogram of extract C, further peaks (1, 2 and 3, Figure 1F) were detected. They could be possibly identified as other phloroglucinols (e.g. furohyperforin and oxyhyperforin), as recently reported (Bergonzi et al 2001). Due to the instability of hyperforin and its tendency to degradation it must be protected from oxidation (Orth et al 1999). Despite its instability, hyperforin is an orally bioavailable component and is present in the plasma of blood samples from subjects after administration of clinically tested hypericum extracts for therapeutic purposes (Biber et al 1998; Chi & Franklin 1999).

Comparison of the antiproliferative effects of *H. perforatum* extracts

We compared the growth-inhibitory effects of extracts in the dark and under the 7.5 J cm^{-2} white light exposure by haematological cell lines (K562, U937) after 48 h, which responded well to the H. perforatum extract in our previous study (Hostanska et al 2002). Growth-inhibitory concentrations 50% (GI50) were evaluated from each individual dose–response curve of all three extracts, A, B and C, simultaneously performed in triplicate and repeated six times. In parallel, the inhibitory activity of rutin and quercetin (Csokay et al 1997; Rong et al 2000), two flavonoid constituents of the extracts, hyperforin and that of phototoxic hypericin (Miccoli et al 1998; Lavie et al 1999) were also investigated. The cytotoxicity of different types of flavonoids showed great variation in their GI50 concentration within the same cell line, and in relation to distinct cell types (Kang & Liang 1997; Cos et al 2001). Quercetin had a more pronounced effect on leukaemia cells than rutin, as shown by the GI50 values, which differed by a factor of ten on the logarithmic scale. At a concentration of approximately 15 μM hyperforin, the growth of both leukaemia cells was reduced by 50%. Light-activated hypericin showed a strong inhibitory effect in contrast to its low effect in the dark. The cytotoxic drug etoposide was used as a positive control in the cytotoxicity assay (Jia et al 2001) and the GI50 concentration for etoposide was found to be 5.8 μM and 0.63 μM for K562 and U937 cells after 48 h of treatment. The GI50

Table 2 GI50 values of H. perforatum constituents in native extracts and of isolated components in leukaemic cell lines.

	K562	U937
Native extract[a]		
A	406.7 ± 50.2	672.5 ± 101.6
B	621.3 ± 113	911.7 ± 143.2
C	248.3 ± 19.4	378.2 ± 43.2
Hypericin[b]		
A	2.2 ± 0.3	3.7 ± 0.5
B	2.6 ± 0.5	3.9 ± 0.6
C	1.6 ± 0.1	2.4 ± 0.3
Hyperforin[b]		
A	24.6 ± 3.0	40.7 ± 5.9
B	25.6 ± 4.7	37.5 ± 5.9
C	1.0 ± 0.1	1.5 ± 0.2
Rutin $3H_2O$[b]		
A	3.6 ± 0.4	5.9 ± 0.9
B	6.1 ± 1.1	10.0 ± 1.4
C	12.5 ± 1.0	19.1 ± 2.2
Quercetin[b]		
A	37.6 ± 4.6	62.2 ± 9.4
B	64.7 ± 11.8	94.9 ± 14.9
C	5.9 ± 0.5	8.9 ± 1.0
Hypericin[c]	37.5 ± 6.3[d]	21.0 ± 2.8[d]
	1.2 ± 0.2[e]	1.0 ± 0.1[e]
Hyperforin[c]	14.2 ± 2.5	15.3 ± 2.1
Rutin $3H_2O$[c]	126.4 ± 16.8	161.5 ± 8.1
Quercetin[c]	28.4 ± 1.96	36.3 ± 4.1
Etoposide	5.8 ± 0.6	0.63 ± 0.1

Data are means ± s.d. [a]Mean concentration values of individual H. perforatum extracts (μg mL^{-1}). [b]Corresponding mean concentration values of single constituents in H. perforatum extracts (μM). [c]Mean concentration values of commercially available compounds (μM). [d]Experiments in the dark. [e]Experiments under 7.5 J cm^{-2} white light.

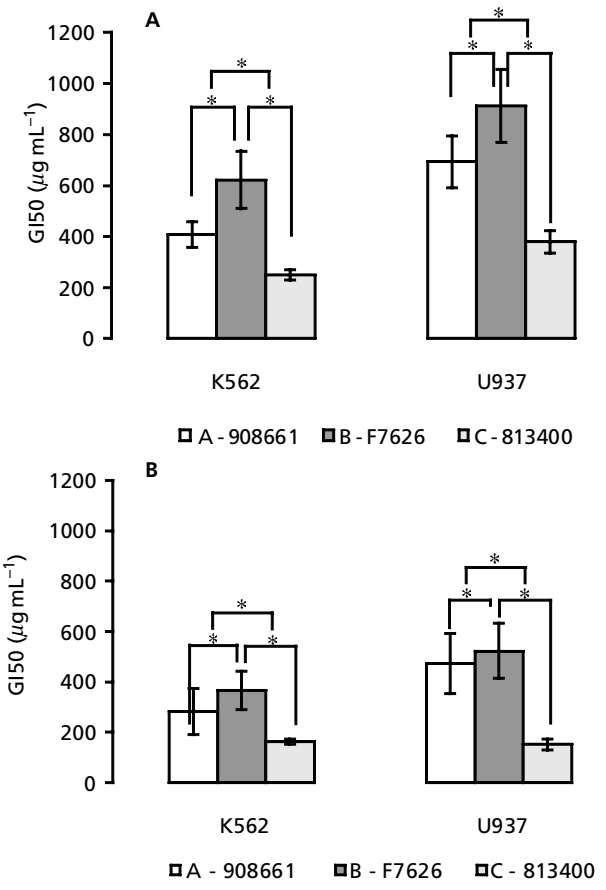


Figure 2 In-vitro growth-inhibitory activity of individual H. perforatum extracts investigated in darkness (A) or under 7.5 J cm^{-2} light activation (B) in leukaemia cell lines K562 and U937. Vertical bars represent means ± s.d. of GI50 from six independent experiments. The statistical evaluation of differences between columns are indicated (*$P < 0.001$, one-way analysis of variance).

values of single compounds are summarized in Table 2. The comparison of GI50 values of individual extracts (Figure 2) showed that there are significant differences in the growth-inhibitory activity between extracts A and B ($P < 0.001$, K562; U937), between B and C ($P < 0.001$, K562; U937) and further between A and C ($P < 0.001$, K562; U937) without light exposure. The individual GI50 values (μg mL^{-1}) of extracts A, B and C were 406.7, 621.3 and 248.3, respectively, for K562 and 672.5, 911.7 and 378.2 for U937 cells. The corresponding values of the three main groups were 1.6–3.9 μM naphthodianthrones, 1.0–40.7 μM phloroglucinols and 30.5–68.5 μM flavonoids (calculated as rutin). The phototoxicity of H. perforatum extract C on both tumour cell lines (162.4 μg mL^{-1} in K562; 150.8 μg mL^{-1} in U937) differs significantly ($P < 0.001$) from that of A (281.5 μg mL^{-1}, for K562; 453.3 μg mL^{-1}, for U937) and B (365.1 μg mL^{-1}, for K562; 522.5 μg mL^{-1}, for U937) after light exposure. The corresponding calculated concentrations of components hypericin, hyperforin, rutin and quercetin to GI50 values of individual native extracts are summarized in Table 2. The growth-inhibitory effects and the differences that extract A and B exert without light activation could be explained by their different hyperforin concentration. The GI50 values of hyperforin in the extracts (24–40 μM) were in a comparable range to the GI50 values of single isolated constituent (15 μM). Both extracts have closely comparable concentrations of the further two main groups of constituents, naphthodiathrones and flavonoids. The flavonoid pattern was similar, because the same herbal mixture and production procedure were employed. In addition, the GI50 values of quercetin in extracts A and B (17.2–42.8 μM) were in accordance with the values found for isolated flavonoid (28.4–36.3 μM). Since hypericum extract C, with the lowest concentration of hyperforin, showed most activity in the same experiments and the corresponding GI50 values of flavonoids quercetin and rutin in the extract were several times lower than that established for isolated substances, the possibility cannot be disregarded that the observed activity was modulated by other component(s) (e.g. procyanidin B2, our unpublished data). The biflavones (Silva et al 1995) and phenylpropanes (chlorogenic and caffeic acids) also showed antiproliferative efficacy toward various malignant cells (Zheng et al 1995; Nagao et al 2001). In addition, the cooperation between individual components of the extract cannot be ruled out. It was reported that the solubility of hypericin was enhanced in the presence of the dimeric procyanidin B2, a constituent of the tannin fraction of H. perforatum extract, which influenced the antidepressant activity exerted in the forced swimming test (Butterweck et al 1998). Recently, it has been shown that the flavonoid rutin is essential for the antidepressant activity of H. perforatum extract (Noldner & Schotz 2002) and that quercetin effectively reduces the toxicity of hypericin (Mirossay et al 2001; Wilhelm et al 2001). Under light exposure, the phototoxicity of the constituent hypericin was decisive, and being in the concentration range of 1–2.5 μM was in accordance with published findings (Lavie et al 1999). However, quercetin at a concentration of 10^{-5} M exerted a protective effect on hypericin-induced cytotoxicity in leukaemia HL-60 cells (Mirossay et al 2001). Lower concentrations of quercetin had no protective effect. The higher potency of extract C (low quercetin content) in comparison with extract A and B (high amount of quercetin) can at least be explained in the light of this finding.

Conclusion

In this study, we showed differences in the antiproliferative activity of H. perforatum extracts, which differ in the concentration of their two main groups of constituents, namely hyperforins and flavonoids. H. perforatum extract contains a number of pharmacologically active constituents, which individually (or in interaction with each other) may potentially participate in its exerted effect. The antiproliferative effect of H. perforatum extract cannot be fully explained by its two main pharmacological constituents, hypericin and hyperforin, as flavonoids at least also contribute to this action. To what extent individual substances

and their combination contribute to the documented growth-inhibitory effect of H. perforatum extracts on leukaemia cells (K562, U937) remains the subject of our current investigations.

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