ANTITUMOUR EFFECT OF PLANT EXTRACTS AND THEIR CONSTITUENTS ON CANCER CELL LINES

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ANOVA	analysis of variance
AO	acridine orange
ATP	adenosine triphosphate
BH	Bcl-2 homology
bp	base pair
cDNA	copy DNA
$CHCl_3$	chloroform
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EB	ethidium bromide
FAR	fluorescence activity ratio
FIC	fractional inhibitory concentration
FITC	fluorescein isothiocyanate
FIX	fractional inhibitory index
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MDR	multidrug resistance
MeOH	methanol
MMLV-RT	Moloney murine leukaemia virus-reverse transcriptase
mRNA	messenger RNA
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
PAMPA	parallel artificial membrane permeability assay
PBS	phosphate-buffered saline
Pgp	P-glycoprotein
PI	propidium iodide
PS	phosphatidylserine
Rh-123	rhodamine 123
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SEM	standard error of the mean

1. INTRODUCTION

Cancer is one of the most prevalent groups of disorders in the population in many countries worldwide. It has been estimated by the American Cancer Society that the death rate from all cancers combined has decreased by 1.5% among men and by 0.8% per year among women since 1993 in the United States, but the cancer incidence rates have stabilized in men and increased by 0.3% per year in women since 1995. Among men, cancers of the prostate, lung/bronchus and colon/rectum account for over 56% of all newly diagnosed cases of cancer. The three most commonly diagnosed cancers among women in 2006 were cancers of the breast, lung/bronchus and colon/rectum, accounting together for about 54% of estimated cancer cases in women [1].

In Hungary, more than 65,000 new cases were reported in 2004 [2]. While the incidence of cancer in Central and Eastern European countries was increasing at an average annual rate of 2.43% in 2001, Western and Nordic countries were undergoing a decrease of 7.27% per year [3]. An overall analysis of cancer mortalities in the European Union ranked Hungary in first place in 2004 [4, 5]. It is the second most common cause of death after cardiovascular diseases.

Cancer is a term describing conditions characterized by uncontrolled cellular proliferation and dedifferentiation [6]. Hanahan and Weinberg identified six hallmark features of the cancer cell phenotype: disregard of signals to stop proliferating and to differentiate; a capacity for sustained proliferation; evasion of apoptosis; and invasion and angiogenesis [7]. The phenotypic changes which the cell undergoes in the process of malignant transformation are a reflection of the sequential acquisition of genetic alterations. This multistep process is not an abrupt transition from normal to malignant growth, but may take place over many years. The mutation of critical genes, including suppressor genes, oncogenes and genes involved in deoxyribonucleic acid (DNA) repair, leads to genetic instability and a progressive loss of differentiation.

The principal modalities of therapy, i.e. surgery, radiotherapy and chemotherapy, may be utilized separately or in combination. Chemotherapy has been used in cancer treatment for more than 50 years, and has proved to be a very efficient strategy. Chemotherapeutics is similar to antibiotics in one property: both can kill certain living cells in the host organism. A basic expectation of antitumour agents is that their effect should be limited to cancer cells. The selective toxicity of the currently used

agents is dependent on the rate of proliferation. In many cases, this selectivity is not adequate, resulting in low activity on slowly growing tumour tissues. On the other hand, a toxic effect on physiological rapidly dividing tissues frequently develops, including bone marrow suppression, deterioration of the gastrointestinal mucosa and alopecia. From these considerations, it seems obvious that an ideal anticancer drug would aim exclusively at the tumour cell instead of utilizing the high growth rate as a marker of targeted cells. Such agents would kill even slowly growing cancer cells without deteriorating normal tissues with a high turnover.

1.1. The search for new agents

Natural products are playing a rapidly increasing role in the lead-finding of candidates for the development of chemotherapeutic agents [8, 9]. They offer a valuable source of compounds with a wide variety of chemical structures with biological activities, and provide important prototypes for the development of novel drugs [10-12]. It is difficult to overrate the importance of natural extracts as potential sources of new drugs. It is estimated that the plant kingdom comprises about 250,000 species, of which approximately 6% have been studied for biological activity, and about 15% have been studied phytochemically [13]. It seems logical that an unguided screening could result in promising hit compounds, but the efficacy of a random search should be far from optimum.

The discovery of cytotoxic agents from natural sources by following up ethnomedicinal uses or the results of previous antitumour screening has had a rich and fruitful past, with the identification of novel agents such as taxoids, campthotecine, podophyllotoxin derivatives and *Vinca* alkaloids [14]. Different methods have been employed in the search for active constituents from plants. They include bioassay-guided isolation, isolate and assay, and biochemical combinatorial chemistry approaches [15]. The bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods, with results obtained from biological testing. The process begins with the testing of an extract to confirm its activity, and further fractionation is carried out on the fractions that are determined to be active. The process of fractionation and biological testing is repeated until a pure compound is obtained.

Plant extracts can be regarded as chemical libraries of structurally diverse compounds, therefore constituting a promising approach in drug discovery. Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural products [16]. Besides anticancer agents, the only other natural compounds whose development is of similarly high interest are the antibacterials (72%); the importance of other groups is much lower [17].

The following natural sources were chosen as potential sources of new anticancer agents:

1.1.1. The Asteraceae family

The Asteraceae family comprises 1310 genera with about 13,000 species worldwide. Asteraceae are commonly used in folk medicine: approximately 300 species have been reported to have ethnomedical uses for the treatment of cancer [18, 19]. Numerous experimental studies have demonstrated that some Asteraceae species exert their antitumour activity due to the presence of flavonoids, sesquiterpene lactones, lignans, acetylenes, triterpenes or glycolipids [20-22]. Many of our tested plants have been documented as anticancer drugs used in folk medicine without identification of their active substances.

1.1.2. *Tamus communis*

Tamus communis L. (black bryony, Dioscoreaceae) is a climbing plant with large tubers which causes irritation when rubbed on the skin [23]. Both the rhizomes and the berries have a reputation as effective rubefacients in folk medicine and they have therefore traditionally been used in several countries for the treatment of rheumatism, arthrosis, lumbago and some dermatoses [24]. Moreover, different parts of the plant have been applied in traditional medicine for the treatment of polyps and tumours [25]. Extracts from *T. communis* display antiviral, anti-inflammatory and analgesic effects in biological systems, but their anticancer activity has not been examined [26, 27].

T. communis contains different active substances, including phenanthrenes [28]. The structurally similar *cis*-stilbene combretastatins, isolated from the African willow tree, *Combretum caffrum* Kuntze (Combretaceae), exhibit potent anticancer activity [29, 30]. The most potent member, combretastatin A-4 in sodium phosphate prodrug form, has recently completed phase I clinical trials as an anti-angiogenic tubulin-binding agent and in non-small-cell lung cancer and cervix carcinoma, and is currently being evaluated in a phase II trial concerning ovarian, thyroid, gastric and other solid tumours [31-36].

1.1.3. Acridone alkaloids

Acridone alkaloids constitute a small group of natural products found exclusively in the family Rutaceae. They are known to exhibit antiviral, antimalarial, antileishmanial, antifungal and photosensitizing activity [37-42]. They also have cytotoxic effects, which are presumed to be exerted by the inhibition of an intracellular enzyme, topoisomerase II, and the inhibition of telomerase by acridine derivatives has also been reported [43, 44]. Acridones fused with a further heterocyclic ring are especially interesting compounds. One of the best-characterized pyranoacridones is acronycine, which has been found to be active against many tumours *in vitro*; this molecule reached phase I-II clinical trials in patients with multiple myeloma, but the results were marginal, probably because of the moderate potency and poor water-solubility of this drug [45, 46]. A clear structure–activity relationship has been reported for this group of compounds, which have been used for the synthesis of further and highly potent antitumour compounds [47, 48]. The new benzo[b]acronycine derivative S23906-1 was selected for further preclinical evaluation [49].

1.2. Apoptosis

Cell death occurs by two alternative and basically different modes: necrosis and apoptosis. Necrosis, the ”ordinary” cell death with the characteristics of a passive process, is traditionally associated with inflammation, occurs in response to severe forms of physical or chemical types of injury, or results from severe depletion of cell energy and nutrition stores [50]. Apoptosis (or programmed cell death) is a fundamental and complex biological process that enables an organism to kill and remove unwanted

cells during any stage of development, therefore maintaining normal homeostasis and eliminating infected or malignant cells. In the experimental development of new anticancer agents, apoptosis is considered the pre-eminent form of pathophysiological cell death [51].

Apoptosis is one of the most important regulatory functions whereby a living organism maintains homeostasis. In some diseases, this regulation is damaged, and the rate of apoptosis is pathologically increased (e.g. neurodegenerative diseases) or decreased (e.g. cancer).

1.2.1. Morphological hallmarks of apoptosis

The definition of apoptosis was first based on a distinct sequence of morphological features observed by electron microscopy, described by Kerr and colleagues in 1972 [52]. The onset of apoptosis is characterized by shrinkage of the cell and the nucleus, and by the condensation of nuclear chromatin into sharply delineated masses that become marginated next to the nuclear membranes. Later the nucleus progressively condenses and breaks up. The term budding has been coined for a process whereby the extensions separate and the plasma membrane seals to form a separate membrane around the detached solid cellular material. These apoptotic bodies are crowded with closely packed cellular organelles and fragments of the nucleus. The apoptotic bodies are rapidly phagocytosed into neighbouring cells, including macrophages and parenchymal cells. Apoptotic bodies can be recognized inside these cells, but eventually they become degraded. If the fragmented cell is not phagocytosed, it will undergo degradation, which resembles necrosis, in a process called secondary necrosis [53]. Apoptotic shrinkage, disassembly into apoptotic bodies and engulfment of individual cells characteristically occur without associated inflammation; the release of intracellular contents into the tissues is therefore prevented.

1.2.2. Biochemical hallmarks of apoptosis

Biochemical features associated with apoptosis include internucleosomal cleavage of DNA, leading to an oligonucleosomal ”ladder”, phosphatidylserine (PS) externalization and proteolytic cleavage of a number of intracellular substrates [54-56].

Activated nucleases are responsible for DNA degradation, resulting in 180-200 base pair (bp) fragments [57]. As a consequence, some of the cells contain a decreased amount of DNA and can be detected as a subdiploid population in the cell cycle distribution by flow cytometry.

In the early stages of apoptosis, plasma membrane alterations occur at the cell surface, and PS translocates from the inner side of the plasma membrane to the outer layer. PS and phosphatidylethanolamine are actively confined to the inner cytofacial leaflet of the plasma membrane by the aminophospholipid translocase. This has been identified as a trigger for stimulation of the phagocytosis of apoptotic cells by macrophages, thus preventing secondary necrosis and inflammation of the surrounding tissue [58]. Surface PS is detectable by flow cytometry using fluorescein isothiocyanate (FITC)-labeled annexin V, a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS [59].

Caspases

Apoptosis occurs through the activation of caspases, a family of cysteinyl aspartate–specific proteases, present as inactive precursors in growing cells [60]. These proteins are distinct from other proteases because they use a cysteine for catalysis and only cleave substrates at Asp-Xxx bonds [61]. Caspases are synthesized as zymogens with an N-terminal prodomain of variable length followed by a large subunit (p20) and a small subunit (p10). During activation, each polypeptide chain is cleaved into a large and a small subunit, which then dimerize. The mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites. [62].

In apoptosis, caspases function in both cell disassembly (as effectors) and in initiating this disassembly in response to proapoptotic signals (as initiators). At least 14 different caspases have been identified in mammalian tissues [63]. One of the best-characterized members is caspase-3, which is activated by other caspases and which can cleave many cellular targets, resulting in the acquisition of apoptotic morphology. Activation of this caspase generally results in an irreversible commitment to cell death [64]. Caspases are responsible for cleaving numerous cellular targets, including structural elements, nuclear proteins and signalling proteins, leading to the morphological hallmarks of apoptosis [62].

The Bcl-2 family

The mitochondria play a pivotal role in the apoptosis of mammalian cells. The permeability of the mitochondrial membrane during apoptosis is regulated directly by the Bcl-2 protein family. This group of proteins, named after a gene involved in follicular B-cell lymphoma, contains at least 19 members with the basic function of inhibiting or promoting apoptosis [65]. The major function of the Bcl-2 family members is to control mitochondrial membrane permeability directly and thereby regulate the release of apoptogenic factors from the intermembrane space into the cytoplasm, leading to apoptosis [66-69]. The Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains, designated BH1, BH2, BH3 and BH4 [70]. Anti-apoptotic members such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1, NR-13 and Boo, which promote cell survival, contain at least BH1 and BH2. Members from the pro-apoptotic family, such as Bax, Bok and Bak, share sequence homology in BH1, BH2 and BH3. Other pro-apoptotic, ”BH3-only” members, such as Bid, Bad, Bik, Blk, Hrk, Bnip3, Bim_L and Noxa, promote cell death.

Activation of the pro-apoptotic proteins, initially localized to the cytosol or the cytoskeleton, is recognized as a key event leading to the alteration and formation of mitochondrial membrane pores and the release of cytochrome *c* [66]. Anti-apoptotic members found in the membrane of the mitochondria, the endoplasmic reticulum or the nuclear membrane, are ”guarding the mitochondrial gate” [71]. It is therefore the ratio of pro- and anti-apoptotic regulatory factors that determines the survival or the death of the cells after harmful stimulation.

1.3. Multidrug resistance

The multidrug resistance (MDR) of tumour cells to chemotherapeutic agents is a major problem in the clinical treatment of cancer. An important mechanism of acquiring the MDR phenotype in mammalian cells is the enhanced expression of a membrane-associated transport protein such as P-glycoprotein or Pgp [72]. Pgp is a member of the highly conserved superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) proteins that constitute one of the largest families in the animal kingdom. To date, at least 49 human ABC proteins have been described, a few of which can confer MDR, and the most examined member of the family is Pgp, also called ABCB1 [73]. The 170

kDa molecular weight Pgp, coded by the MDR1 gene, rapidly extrudes hydrophobic antitumour drugs from target cancer cells and alters the subcellular drug distribution. Pgp has 12 transmembrane regions and two ATP-binding sites. Two ATP hydrolysis events are needed to transport one drug molecule. This protein was identified first in Chinese hamster ovary cells by Juliano and Ling, who detected a carbohydrate-containing glycoprotein in the surface of colchicine-resistant hamster cells [74]. This protective response of tumour cells decreases the effectiveness of many chemotherapeutic agents [75].

The physiological role of Pgp in the organs is to afford protection against toxic insults via specialized excretory, secretory and barrier functions [76, 77]. Pgp is widely expressed in the epithelial cells of the intestine, liver, and kidney and in the endothelial cells of the brain and placenta.

In general, agents used to antagonize MDR alter the drug accumulation defect present in MDR cells. Altough a variety of agents have been tested in the search for chemosensitizers to inhibit MDR, there is no useful agent in chemotherapy to date. First-generation modulators were not specifically developed for the inhibition of MDR; they were discovered by chance (verapamil, cyclosporin A, reserpine, etc.) [78]. The unacceptable toxicity characteristic of the first-generation molecules was eliminated in the second-generation agents, which were designed after chemical modification of the earlier agents. These modulators have better tolerability, but undergo unpredictable pharmacokinetic interactions (the *R* isomer of verapamil, valspodar, an analogue of cyclosporin D, biricodar, etc.). Third-generation compounds (tariquidar, zosuquidar, laniquidar, etc.) were designed so as to lack other pharmacological effects and to confer greater selectivity and specificity for Pgp. Several compounds from this group are currently undergoing clinical trials [79].

2. SPECIFIC AIMS

The main aims of the present project were to search for natural products that exhibit activity against tumour cells, including a cytostatic effect, and that potentiate currently used anticancer drugs.

- The first step in our studies was an *a priori* selection, based partly on literature surveys, of plants for antiproliferative investigation. Species of the Hungarian Asteraceae family were subjected to a systematic screening for cytostatic activity.
- The purpose of the further work was the identification of the pure compounds responsible for the detected cytotoxic activity of the extracts. Extracts of *T. communis* were chosen for a complete bioassay-guided isolation of effective components.
- Beside the search for new chemical entities, we targeted the development of previously isolated, but not investigated natural compounds. We focused on a description of the mechanism of the cytostatic effects of acridone alkaloids from *Ruta graveolens*.
- An additional aim of our work was to explore substances with the ability to inhibit Pgp-mediated MDR and to clarify their potential importance in a set of drug combination experiments.

3. MATERIALS AND METHODS

3.1. Plant material

The initial set of Asteraceae plants were collected in the flowering period, between June and August 2004, from several regions of Hungary. From the air-dried, powdered plant material, extracts were prepared with methanol (MeOH), followed by filtration and evaporation to dryness under reduced pressure. The residues were dissolved in 50% aqueous MeOH and were subjected to solvent-solvent partition between *n*-hexane (**extract A**), chloroform ($CHCl_3$) (**extract B**) and water (**extract C**). After extraction with MeOH, the residual plant materials were dried and extracted with boiling water. The filtered extracts were freeze-dried, affording **extract D**.

In the case of *T. communis*, the rhizomes were collected in the Mecsek Hills (Hungary) in June 2003. The effective agent was determined by bioassay-guided fractionation. First, the rhizomes were percolated with MeOH and the resulting solution was partitioned with $CHCl_3$. The $CHCl_3$ fraction was chromatographed by vacuum liquid chromatography on silica gel, yielding 13 main fractions. After further different chromatographic steps, compounds were isolated and then identified.

The acridone alkaloids (isolated from the roots and herbs of *Ruta graveolens* L., common rue, Rutaceae) were obtained as pure compounds (arborinine (**1**), evoxanthine (**2**), isogravacridone chlorine (**3**), rutacridone (**4**), gravacridonediol (**5**), gravacridonetriol (**6**), gravacridonediol monomethyl ether (**7**), gravacridonetriol monoglycoside (**8**) and gravacridonediol monoglycoside (**9**) (**Fig. 1**)) [80-83].

3.2. Tumour cell lines

MCF7 (breast adenocarcinoma, ATCC: HTB-22), HeLa (cervix adenocarcinoma, ATCC: CCL-2) and A431 (skin epidermoid carcinoma, ATCC: CRL-1555) cells were cultivated in minimum essential medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids and antibiotic-antimycotic solution. L5178 mouse T-cell lymphoma cells (L5078-R, ATCC: CRL-1722) were transfected with pHa MDR1/A retrovirus, as previously described [84]. The parent cells and the human MDR1-transfected L5178 mouse T-cell lymphoma subline were cultured in

McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. MDR1 gene-expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. The cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

3.3. MTT assay

Antiproliferative effects were measured *in vitro* by using MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assays [85]. Antiproliferative and cytotoxic effects were distinguished by cell number and the duration of treatment (72 h, 5000 cells/w, and 24 h, 25000 cells/w, respectively) [86]. After incubation, the living cells were assayed by the addition of 20 µl of 5 mg/ml MTT solution. Finally, the reduced MTT was assayed at 545 nm; wells with untreated cells were utilized as controls. All *in vitro* experiments were carried out on 2 microplates with at least 5 parallel wells. Stock solutions of the tested materials (10 mg/ml for the extracts, 30 mM for the pure compounds) were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation. Extracts which demonstrated potent activity (growth inhibition >50%) were selected for further *in vitro* testing (IC_{50}, calculated from dose-response curve and cytotoxicity).

To study the interactions between acridones and doxorubicin, the checkerboard method was applied [87-89]. A series of 2-fold dilutions of the acridones was tested in combination with 2-fold dilutions of doxorubicin. The dilutions of doxorubicin (A) were made in a horizontal direction and of the resistance modifiers (B) vertically in the microtitre plate. The cell growth rate was determined after MTT staining. Drug interactions were evaluated according to the following system (fractional inhibitory concentration = FIC; fractional inhibitory index = FIX):

$FIC_A = IC_{50A\ in\ combination} / IC_{50A\ alone}$	FIX < 0.5	Synergism
$FIC_B = IC_{50B\ in\ combination} / IC_{50B\ alone}$	FIX = 0.51-1	Additive effect
$FIX = FIC_A + FIC_B$	1 < FIX < 2	Indifferent
	effect FIX > 2	Antagonism

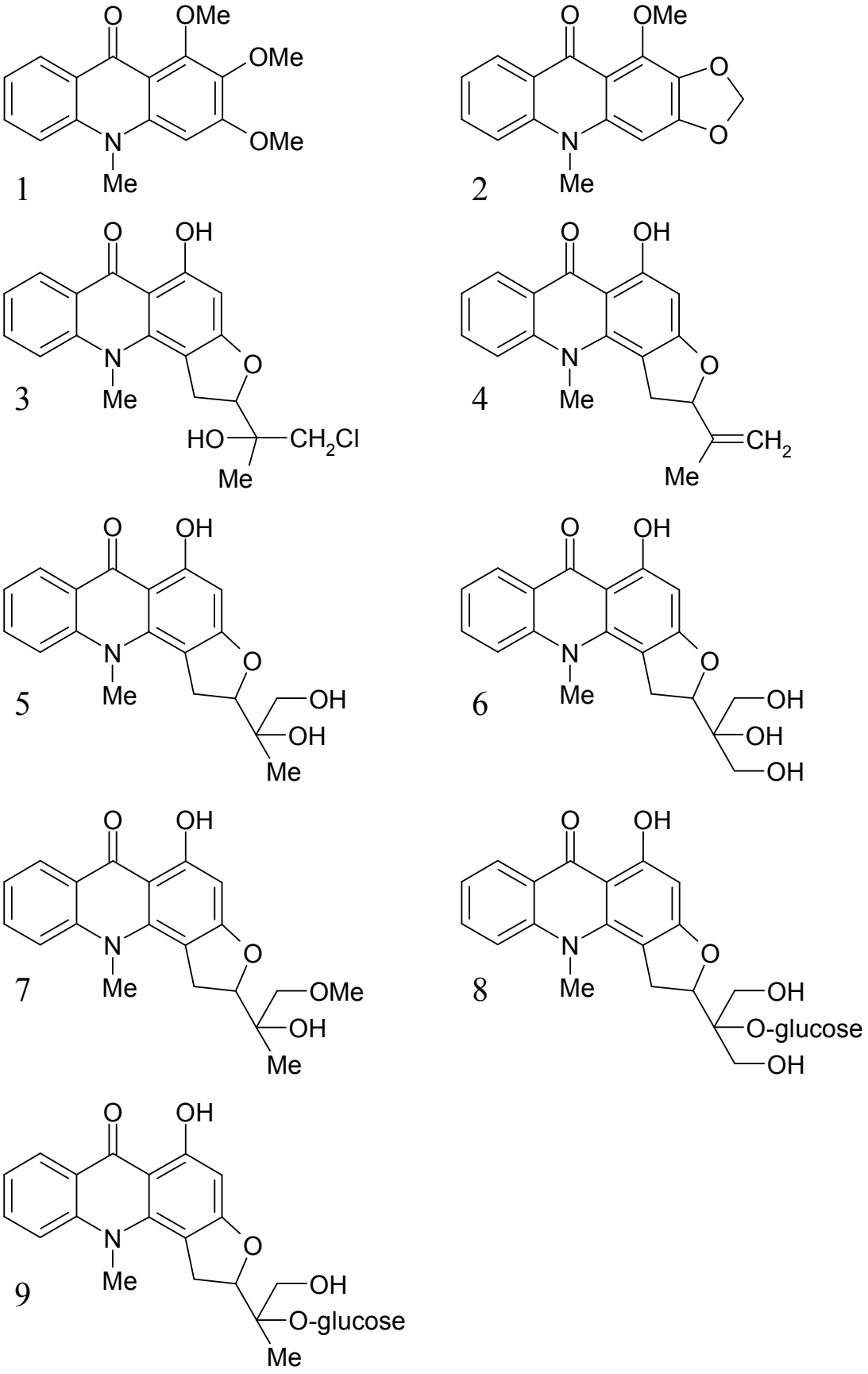


Figure 1. Chemical structures of the acridone alkaloids.

3.4. Transmembrane permeability assay

The transmembrane permeabilities were determined in parallel artificial membrane permeability assay (PAMPA) and the effective permeability values (log P_e) were calculated as published earlier [90]. A MultiScreen filter plate was used with 15 μl of a 5% solution of *n*-dodecane in *n*-hexane as lipid layer and the concentration of the tested acridone alkaloid in the acceptor plate was determined with a microplate reader at 340 nm after incubation for 5 h.

The log P_e was calculated from the ratio of the absorbance of compounf found in the acceptor chamber divided by the theoretical equilibrium absorbance:

$$\log P_e = \log\left\{ \frac{V_D \times V_A}{(V_D + V_A) \times A \times t} \times -\ln\left(1 - \frac{CC_{acceptor}}{CC_{equilibrium}}\right)\right\}$$

where V_D and V_A is the volume of the acceptor and donor compartment (0.15 and 0.3 cm^3), respectively, A is the accessible filter area (0.24 cm^2) and t is the incubation time (300 min). $CC_{acceptor}$ is the compound concentration in the acceptor compartent and $CC_{equilibrium}$ is the equilibrium concentration (240 μM).

3.5. Ethidium bromide and acridine orange dual staining

Staining with ethidium bromide (EB) and acridine orange (AO) was carried out in a 96-well plate format after 24-h acridone treatment to visualize the basic morphological events of apoptosis [91]. This staining allowed the identification of intact, early-apoptotic, late-apoptotic and necrotic cells. AO permeates all cells and makes the nuclei appear a homogeneous green for live cells and spotted green for early-apoptotic cells. EB is taken up by cells only when the cytoplasmic membrane integrity has been lost; it stains the nucleus red. Plates were centrifuged at 1000 rpm, and 8 μl of staining solution was added to each well (0.1 mg/ml for both AO and EB in phosphate-buffered saline (PBS)). After 10 min, the wells were washed with PBS and the cells were viewed with a Nikon Eclipse inverted microscope at 200x magnification with a 500/20 nm excitation filter, a cut-on 515-nm LP dichromatic mirror and a 520-nm LP barrier filter (Chroma Technology; Rockingham, VT, USA). Pictures were taken with a Nikon Coolpix 4500 digital camera.

3.6. Flow cytometric analysis

For the measurement of cellular DNA content, flow cytometric analysis was used after 24-h treatment. HeLa cells were washed with PBS and permeabilized by detergent treatment (0.1% Triton X-100 in PBS) for 30 min. DNA was stained with propidium iodide (PI) (10 μg/ml) in the presence of RNase (50 μg/ml). The samples were then analysed by FACStar (Becton-Dickinson; Mountain View, CA, USA). In each analysis, the percentages of the cells in the different cell-cycle phases (G1, S and G2/M) were calculated by using winMDI2.8. The subdiploid subG1 fraction was regarded as the apoptotic cell population [92].

For the reversal of MDR, the L5178 parent and MDR cells (10^6/ml) were incubated for 10 min at room temperature with one or other of the acridone alkaloids. Next, 10 μl of the indicator rhodamine 123 (Rh-123; 1 mg/ml) was added to the samples and the cells were incubated for 20 min at 37 °C, washed twice and resuspended in 0.5 ml of PBS for flow cytometric analysis. Verapamil (final concentration 40.6 μM) was used as a positive control in the Rh-123 exclusion experiments. The results of the test were calculated for the treated MDR and parental cell lines as compared with the untreated cells. The fluorescence activity ratio (FAR) was calculated as the ratio of the fluorescence of the treated MDR and untreated cells.

3.7. Reverse transcriptase-PCR studies

The effects of the tested compounds on the messenger ribonucleic acid (mRNA) expression pattern of Bax, Bcl-2 and MDR1 were determined by a reverse transcriptase-polymerase chain reaction (RT-PCR) technique. After treatment (24 h for HeLa, and 48 h for L5178 MDR cells), 10^6 cells were treated with denaturing solution [93]. After precipitation with isopropanol, the RNA was washed with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 μl DNase- and RNase-free distilled water and the RNA concentrations were determined spectrophotometrically. 0.5 μg of RNA was denatured at 70 ºC for 5 min in a reaction mixture containing 20 μM oligo(dT), 20 U of RNase inhibitor, 200 μM deoxynucleotide triphosphate (dNTP) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM $MgCl_2$ in a final reaction volume of 20 μl. After the mixture had been cooled to 4 ºC, 20 U of Moloney murine leukaemia virus-reverse

transcriptase (MMLV-RT) and RNase H Minus were added and the mixture was incubated at 37 ºC for 60 min. The PCR was carried out with 5 µl of copy DNA (cDNA), 25 µl of ReadyMix REDTaq PCR reaction mix, 2 µl of sense and antisense primer and 16 µl of DNase- and RNase-free distilled water with a PCR Sprint thermal cycler. Human and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal control [94, 95]. The products were separated on 2% agarose gels, stained with ethidium bromide and photographed. The sequences of the oligonucleotide primers for Bcl-2, Bax and MDR1 were the same as previously reported (**Table 1**) [96-98].

Gene		Primers	Size	Ref.
Bax	forward	TGGCAGCTGACATGTTTTCTGAC	195 bp	[97]
	reverse	CGTCCCAACCACCCTGGTCT		
Bcl-2	forward	GACTTCGCCGAGATGTCCAG	225 bp	[96]
	reverse	CAGGTGCCGGTTCAGGTACT		
MDR1	forward	GCCTGGCAGCTGGAAGACAAATACACAAAATT	283 bp	[98]
	reverse	CAGACAGCAGCTGACAGTCCAAGAACAGGAT		
Mouse GAPDH	forward	GGTGAAGGTCGGTGTGAACGGATT	501 bp	[95]
	reverse	ATGCCAAAGTTGTCATGGATGACC		
Human GAPDH	forward	TGAACGGGAAGCTCACTGG	307 bp	[94]
	reverse	TCCACCACCCTGTTGCTGTA		

Table 1. Sequences of the primers.

3.8. Statistical analysis

The results of the cytostatic and cytotoxic assays are presented as means ± standard error of the mean (SEM) from 5 parallel samples in two independent experiments. All of the other experiments (PAMPA assay, flow cytometry and RT-PCR studies) were performed in triplicate. Differences between the results of different treatments were analysed by analysis of variance (ANOVA) followed by the Newman-Keuls *post hoc* test. All statistical analyses were carried out with GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, USA).

4. RESULTS

4.1. Determination of the antiproliferative activity of plant extracts

4.1.1. Asteraceae screening

Species from the Asteraceae family were selected for this study, partly as a follow-up of ethnomedicinal uses, and partly randomly selected. From a set of 25 plant species of the four tribes [Astereae (6), Inuleae (3), Heliantheae (5) and Anthemideae (11)], 228 different extracts were prepared with *n*-hexane (**A**), $CHCl_3$ (**B**), aqueous MeOH (**C**) and H_2O (**D**) from selected plant organs. These extracts were initially screened on three cancer cell lines at 10 μg/ml (data not shown). 25 extracts that exerted ≥50% inhibition of proliferation on any cell line were further investigated. For the selected extracts, IC_{50} values were determined; direct cytotoxic and cytostatic effect were distinguished at 30 μg/ml (**Table 2**).

Among the extracts, mainly fractions B (72% of the selected extracts), containing $CHCl_3$-soluble lipophilic constituents, were found to be active. In some cases, the *n*-hexane and aqueous-MeOH extracts (fractions A and C, respectively) were also effective. None of the residual aqueous extracts (fraction D) demonstrated a pronounced antiproliferative effect (>50% inhibition of proliferation) against any cell line.

For the active Anthemideae species, the extracts of the aerial parts proved to be active (*A. collina*, *A. ruthenica*, *A. asiatica* and *A. japonica*). Several extracts exhibited considerable IC_{50} values and the extracts of *A. collina* displayed some selectivity for dividing cells as evidenced by the high cytostatic-cytotoxic differences.

For the Astereae plants (*E. canadensis* and *E. annuus*), the extracts from the roots seemed to be more effective than those from other organs and the MCF7 cells were slightly more sensitive than the other two cell lines, as demonstrated by the IC_{50} values. The *n*-hexane extracts of the roots of *E. canadensis* exhibited the highest activity in the tribe (IC_{50} = 3.32 μg/ml), but this extract (similarly to most extracts from this tribe) displayed substantial direct cytotoxic action.

In the Heliantheae tribe, noteworthy antiproliferative activities were recorded for *A. artemisiifolia*, *H. annuus* and *X. italicum*, in agreement with the previously published

ethnomedicinal data. The most potent extracts in the whole screen were the $CHCl_3$ extracts of *H. annuus* and *X. italicum*, which had IC_{50} values <5 μg/ml on all cell lines. The $CHCl_3$ extracts of *X. italicum* exhibited excellent antiproliferative action and some of them proved to be selective towards the growing cells (e.g. the aqueous MeOH extracts of the buds/flowers).

Table 2. Antiproliferative IC_{50} values (μg/ml), antiproliferative and cytotoxic activities (% ± SEM) of the selected plant extracts (30 μg/ml; exposure time 24 or 72 h, respectively) against human cancer cell lines and the difference of these two values.

Species	Plant parts	Solvent and cell line		IC_{50}	Cytostatic activity	Cytotoxic activity	Difference
ANTHEMIDEAE							
Achillea collina	flowers	B	HeLa	2.89	94.16 ± 0.48	24.86 ± 1.46	69.30
			MCF7	>30	53.28 ± 2.33	-3.22 ± 7.98	56.50
			A431	17.49	95.10 ± 0.19	29.10 ± 2.71	66.00
	folium	B	HeLa	2.02	96.97 ± 0.45	42.79 ± 1.81	54.18
			MCF7	8.51	69.95 ± 3.31	5.464 ± 2.25	64.48
			A431	>30	50.23 ± 3.25	20.98 ± 2.54	29.25
	herba	B	HeLa	1.74	91.83 ± 1.74	29.27 ± 1.28	62.56
			MCF7	>30	63.42 ± 3.65	0.33 ± 4.52	63.09
			A431	13.68	96.62 ± 0.55	49.22 ± 4.86	47.40
Anthemis ruthenica	herba	B	HeLa	6.75	89.84 ± 0.69	53.79 ± 1.91	36.05
			MCF7	7.34	88.37 ± 0.38	44.17 ± 3.96	44.20
			A431	7.11	94.72 ± 0.74	67.69 ± 2.92	27.03
Artemisia asiatica	flowers	B	HeLa	5.99	98.84 ± 0.20	67.26 ± 4.48	31.58
			MCF7	2.85	101.2 ± 0.21	42.99 ± 5.34	58.21
			A431	4.19	100.5 ± 0.09	52.66 ± 2.00	47.84
	folium	B	HeLa	10.42	96.07 ± 0.62	44.88 ± 1.74	51.19
			MCF7	10.45	97.48 ± 1.03	51.63 ±10.60	45.85
			A431	4.96	99.42 ± 0.51	88.49 ± 1.76	10.93
Artemisia japonica	folium	B	HeLa	9.72	94.57 ± 0.50	45.04 ± 3.01	49.53
			MCF7	6.43	97.97 ± 1.11	54.77 ± 4.72	43.20
			A431	7.95	97.92 ± 0.86	85.01 ± 3.61	12.91
	flowers	B	HeLa	6.89	97.48 ± 0.26	36.05 ± 3.95	61.43
			MCF7	3.01	98.68 ± 0.66	88.34 ± 2.94	10.34
			A431	4.88	99.98 ± 0.13	90.70 ± 2.14	9.28
ASTEREAE							
Erigeron canadensis	herba	A	HeLa	17.4	71.09 ± 1.16	32.23 ± 1.28	38.86
			MCF7	7.93	81.22 ± 1.79	15.32 ± 4.56	65.90
			A431	11.6	72.55 ± 0.86	26.68 ± 0.88	45.87
	herba	B	HeLa	18.72	68.37 ± 2.27	24.20 ± 1.61	44.17
			MCF7	15.8	81.42 ± 0.72	30.29 ± 2.60	51.13
			A431	21.46	59.00 ± 1.40	17.54 ± 1.83	41.46
	roots	A	HeLa	6.47	85.76 ± 1.85	56.76 ± 2.25	29.00
			MCF7	3.32	88.94 ± 0.64	60.46 ± 2.64	28.48
			A431	9.47	86.99 ± 2.15	50.93 ± 0.93	36.06

Table 2 (*continued*)

Species	Plant parts	Solvent and cell line		IC_{50}	Cytostatic	Cytotoxic	Difference
Erigeron annuus	roots	A	HeLa	12.45	95.28 ± 0.19	39.52 ± 2.99	55.76
			MCF7	6.43	95.98 ± 0.57	91.74 ± 1.55	4.24
			A431	20.12	90.97 ± 0.54	48.22 ± 1.39	42.75
	roots	B	HeLa	12.94	88.42 ± 1.34	50.25 ± 0.89	38.17
			MCF7	9.17	95.20 ± 0.07	42.78 ± 1.55	52.42
			A431	13.96	98.06 ± 8.59	46.50 ± 2.43	51.56
HELIANTHEAE							
Ambrosia artemisii-folia	folium	B	HeLa	19.82	60.40 ± 1.09	31.37 ± 1.67	29.03
			MCF7	10.24	75.38 ± 1.54	19.38 ± 1.79	56.00
			A431	11.12	86.17 ± 0.82	32.36 ± 1.74	53.81
	root	A	HeLa	>30	50.25 ± 1.00	40.09 ± 1.20	10.16
			MCF7	>30	47.06 ± 2.93	18.61 ± 1.33	28.45
			A431	8.55	62.78 ± 1.15	34.39 ± 2.36	28.39
Helianthus annuus	roots	B	HeLa	3.51	90.91 ± 1.61	38.77 ± 2.33	52.14
			MCF7	3.36	87.46 ± 2.89	16.11 ± 3.44	71.35
			A431	4.19	97.54 ± 0.51	24.91 ± 3.94	72.63
Xanthium italicum	buds/ flowers	A	HeLa	15.0	92.95 ± 0.28	33.25 ± 4.59	59.70
			MCF7	11.14	95.28 ± 0.32	41.97 ± 5.46	53.31
			A431	6.67	94.88 ± 0.49	44.08 ± 1.47	50.80
		B	HeLa	2.78	99.62 ± 0.80	91.48 ± 2.45	8.14
			MCF7	2.69	99.72 ± 0.51	97.71 ± 0.59	2.01
			A431	0.74	99.53 ± 0.06	98.89 ± 0.57	0.64
		C	HeLa	13.55	85.33 ± 1.39	9.94 ± 2.68	75.39
			MCF7	9.96	95.81 ± 0.38	13.31 ± 5.25	82.50
			A431	7.98	94.06 ± 1.03	18.22 ± 9.10	75.84
	folium	B	HeLa	2.86	94.24 ± 3.28	79.60 ± 2.56	14.64
			MCF7	2.24	99.14 ± 0.67	97.27 ± 0.26	1.87
			A431	0.71	100.4 ± 0.14	96.53 ± 0.43	3.87
	roots	A	HeLa	10.60	93.71 ± 0.27	48.40 ± 2.16	45.31
			MCF7	9.59	94.87 ± 1.08	31.15 ± 3.44	63.72
			A431	9.83	98.00 ± 0.16	52.83 ± 1.28	45.17
		B	HeLa	7.75	92.89 ± 0.57	36.64 ± 3.72	56.25
			MCF7	4.55	96.44 ± 0.35	60.44 ± 2.23	36.00
			A431	5.04	97.68 ± 0.08	59.89 ± 1.46	37.79
INULEAE							
Inula ensifolia	fructus/ flowers	B	HeLa	2.68	90.70 ± 0.79	53.74 ± 2.80	36.96
			MCF7	>30	52.01 ± 4.91	17.72 ± 3.57	34.29
			A431	17.88	85.10 ± 1.00	73.69 ± 1.16	11.41
Telekia speciosa	folium	B	HeLa	4.29	97.03 ± 1.15	63.52 ± 3.86	33.51
			MCF7	5.22	99.60 ± 0.41	58.33 ± 4.16	41.27
			A431	2.93	99.85 ± 0.22	87.54 ± 1.75	12.31
	flowers		HeLa	8.55	98.45 ± 0.27	32.68 ± 4.89	65.77
		B	MCF7	6.78	97.33 ± 2.33	12.06 ± 4.11	85.27
			A431	4.99	90.03 ± 0.63	46.79 ± 3.06	43.24

As concerns the Inuleae species, *I. ensifolia* was found to be moderately active, while *T. speciosa* proved to have marked efficacy.

4.1.2. Bioassay-guided isolation of cytostatic compounds from the rhizome of *T. communis*

The rhizome of *T. communis* was selected on an ethnomedicinal basis for bioassay-guided fractination on HeLa cells using the MTT assay. In the first set of experiments, the $CHCl_3$ extract exhibited pronounced and dose-dependent antiproliferative activity (IC_{50} = 0.02 μg/ml), while the MeOH extract was inactive. For identification of the components responsible for the effect of the extract, it was fractionated into 13 fractions. This led to a differential cytotoxicity profile, in which fractions IV, V+VI, VIII, IX and XIII exhibited >50% activity at a concentration of 10 μg/ml (**Fig. 2**). In further appropriate chromatographic steps, 5 active phenanthrenes were isolated from these active fractions. The structure of the isolated phenanthrenes was determined by UV, MS, NMR and NOESY spectra.

From fraction IV, 7-hydroxy-2,3,4-trimethoxyphenanthrene (**10**) and 2,7-dihydroxy-3,4,8-trimethoxyphenanthrene (**12**) were isolated. 2,7-Dihydroxy-3,4-dimethoxyphenanthrene (**11**) was purified from fractions V-VI, and 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**13**) and 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**14**) from fractions VIII-IX. The cytostatic activities of the isolated compounds were determined on HeLa cells. The IC_{50} values and the structures of the phenanthrenes are listed in **Table 3**. The results of the MTT tests revealed that compounds **10**, **12** and **14** displayed marked cell growth inhibitory activity and the IC_{50} value of **12** proved to be comparable to that of the positive control (doxorubicin IC_{50} = 0.15 μM). Compound **10** is a new natural compound, while the known **11** (nudol), **12** (confusarin) and **13** were described first in *T. communis*. Compound **14** (previosly reported as TaVIII) was earlier isolated from the rhizome of this plant [99, 100].

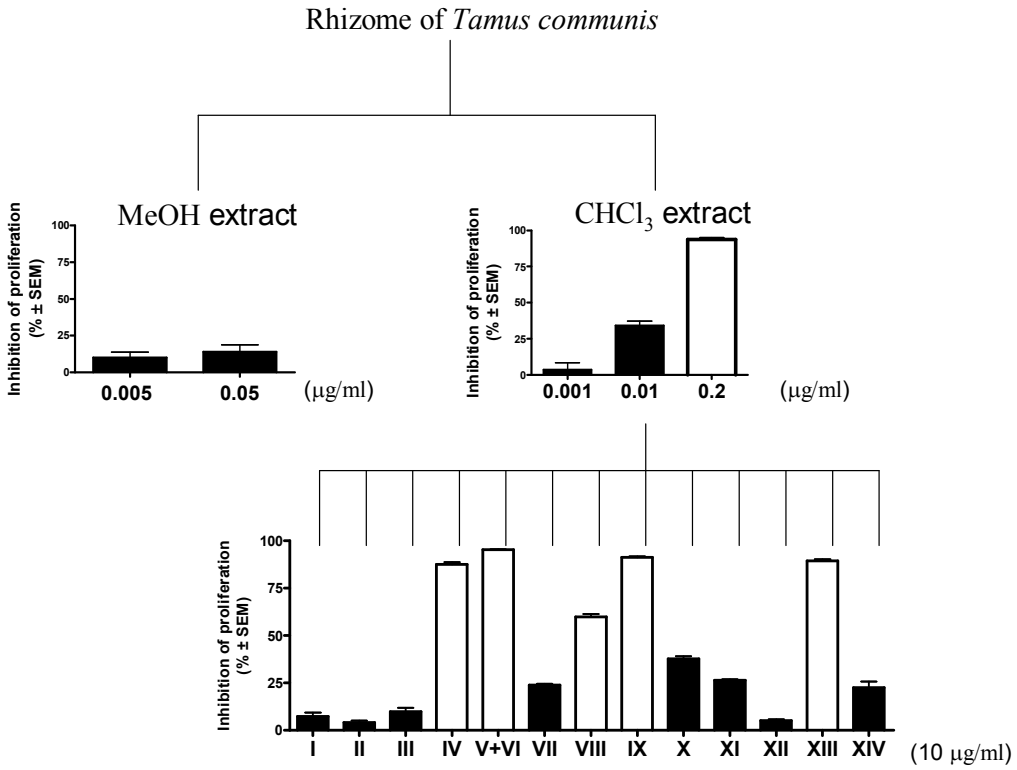
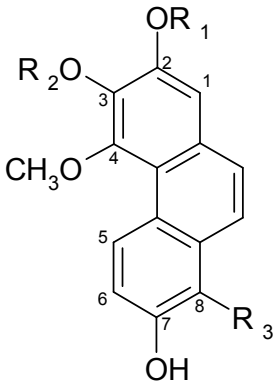


Figure 2. Scheme for the fractionation and the bioassay-guided isolation from *T. communis.*



	10	**11**	**12**	**13**	**14**
R_1	$-CH_3$	-H	-H	$-CH_3$	$-CH_3$
R_2	$-CH_3$	$-CH_3$	$-CH_3$	-H	-H
R_3	-H	-H	$-OCH_3$	$-OCH_3$	-H
IC_{50} (μM)	13.85	20.18	0.97	>30	6.66

Table 3. Structures and cytostatic activities on HeLa cells of the phenanthrenes isolated from *T. communis*.

4.2. Antitumour effect of the acridones

The antiproliferative effects and the permeabilities of the investigated compounds are listed in **Table 4.** Compound **1** proved to be an outstandingly potent cytostatic agent, especially against HeLa cells. Its IC_{50} values are comparable with that of cisplatin; the antiproliferative action of compound **1** was not reported earlier. Compound **2** did not display any substantial activity on any of the cell lines up to the final concentration of 30 μM. The tested furanoacridones (**3-9**) exhibited slightly different sequences of potency against HeLa (**7 > 4 > 5 > 3 > 6 > 8+9**), MCF7 (**3 > 4 > 7 > 5 > 6 > 8+9**) and A431 cells (**3 > 7 > 4 > 5 > 6 > 8+9**). The differences between the IC_{50} values of **3**, **4**, **5** and **7** were not substantial, while **6** and **8+9** were always much less active. The permeabilities of these compounds were characterized by PAMPA, and log P_e values were calculated. The sequence of permeability was found to be **4 > 5 > 3 > 7 > 6 > 8+9**. The polar compounds such as gravacridonetriol and gravacridonediol had lower permeability (lower log P_e) and cytostatic effect, whereas hydrophobic compounds had higher permeability in the PAMPA system. The correlation coefficients between log P_e and IC_{50} were found to be 0.717, 0.856 and 0.578 for HeLa, MCF7 and A431 cells, respectively.

In the further tests, three selected acridones were studied for a description of their mechanisms of action.

Table 4. Calculated IC_{50} and log P_e values of the tested compounds.

Compound	IC_{50} value (mM)			log P_e value
	HeLa	MCF7	431	
1	1.84	11.74	12.95	n.d.
2	not active*	not active*	not active*	n.d.
3	8.35	4.53	3.02	-5.108
4	5.27	7.69	14.41	-4.994
5	7.87	19.91	14.83	-5.087
6	23.24	99.60	27.88	-5.865
7	3.84	13.20	11.97	-5.361
8+9	not active*	not active*	not active*	-6.359
Doxorubicin	0.15	0.28	0.15	n.d.
Cisplatin	12.43	9.63	2.84	n.d.

(Asterisks indicate that the compound did not inhibit cell proliferation to a significant extent at 30 μM. n.d.: not determined.)

Besides the antiproliferative property, effects on the cell cycle profile were additionally characterized by labelling the cellular DNA content with PI. Flow cytometry revealed that **3**, **4** and **7** exerted effects on the cell cycle distribution. The exposure of HeLa cells to compounds **3**, **4** and **7** for 24 h resulted in an increased proportion of cells in the S phase and a decreased proportion of cells in the G_2/M phase as compared with the control. To establish the change in the cell cycle, the quotient of S to G2/M was calculated, which increased in a dose-dependent manner (**Fig. 3**).

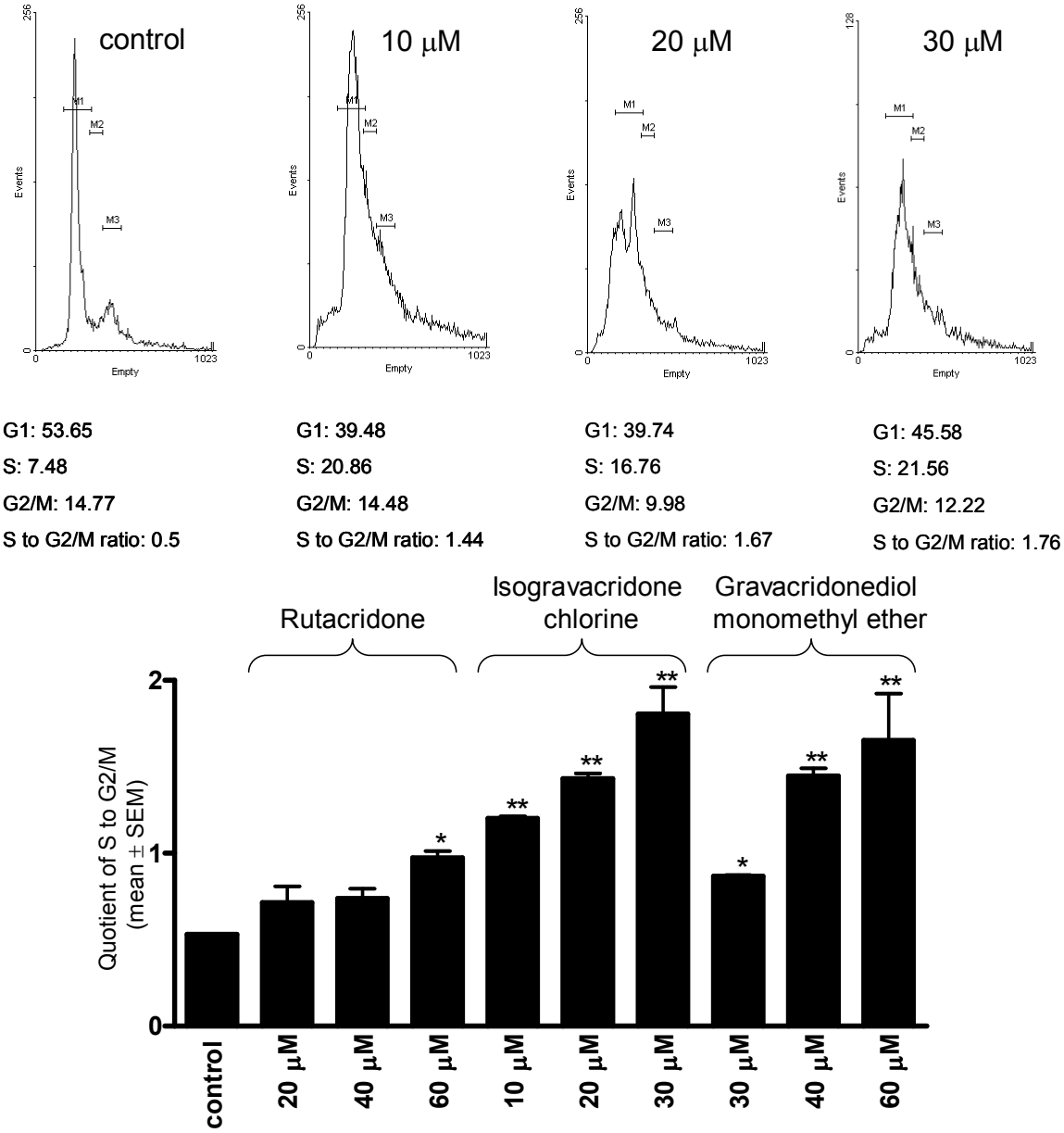


Figure 3. Cell cycle analysis. Effects of isogravacridone chlorine, rutacridone and gravacridonediol monomethyl ether on cell cycle in HeLa cells after 24-h treatment. Upper panel: A representative histogram of isogravcridone chlorine-treated HeLa cells. Lower panel: Effects of isogravacridone chlorine, rutacridone and gravacridonediol monomethyl ether on the quotient of S to G2/M of HeLa cells. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as compared with the control value.

4.2.1. Apoptosis induced by acridone alkaloids

The typical morphological features of apoptosis, including increased cell membrane permeability, cellular shrinkage and granulation in the nucleus, are major consequences of the apoptotic trigger. HeLa cells treated with **3**, **4** or **7** exhibited apoptotic morphological changes, as detected with AO/EB double staining. The numbers of apoptotic cells, including early-apoptotic (AO +, EB –) and late-apoptotic cells (AO +, EB +) were increased in a dose-dependent manner. After treatment with acridones, an increase in cell membrane permeability was observed, as evidenced by the red fluorescence of EB in the nucleus. At low concentrations, a majority of AO +, EB – cells were detected, indicating apoptotic granulation and an intact cell membrane. As the concentration was increased late-apoptotic (AO +, EB +) and necrotic (AO –, EB +) cell populations appeared (**Fig. 4**).

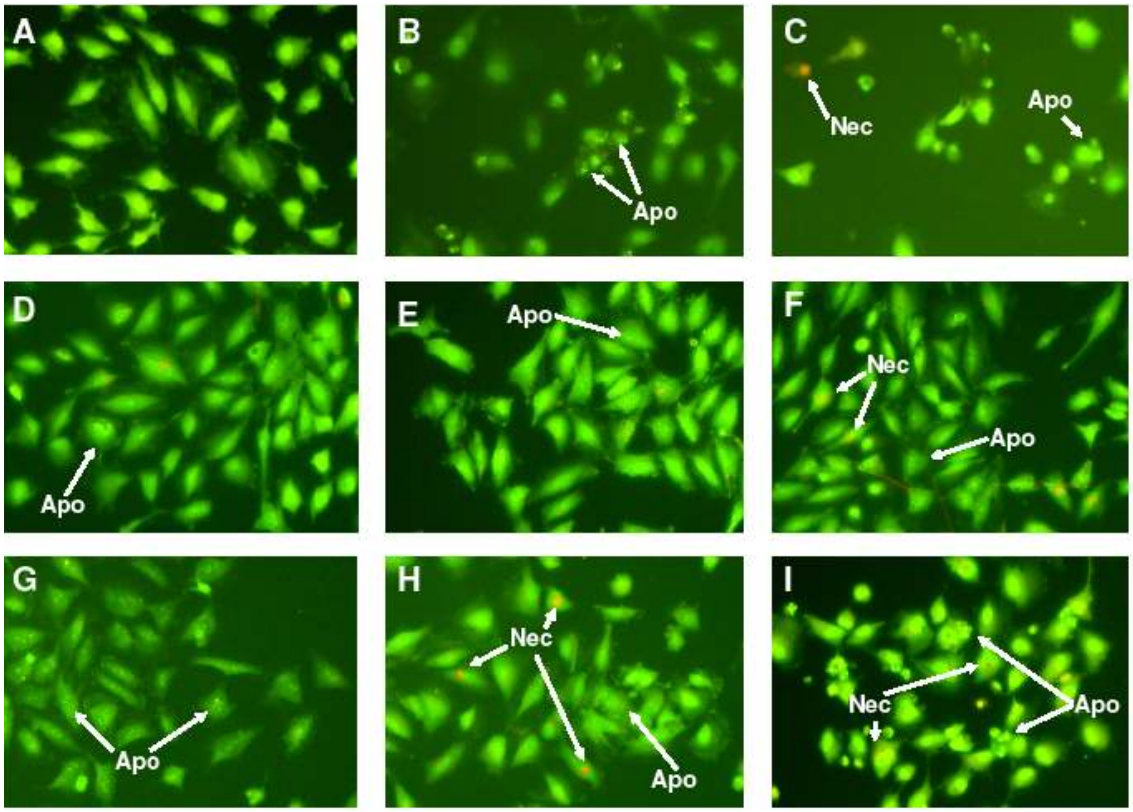


Figure 4. Fluorescent microscopic pictures of double staining with AO and EB. Control HeLa cells (A), treatment with 20 or 40 μM isogravacridone chlorine (B, C); 10, 30 or 60 μM rutacridone (D, E and F); or 10, 20 or 30 μM gravacridonediol monomethyl ether (G, H and I), respectively, for 24 h. Red spots indicate the fluorescence of EB; yellow spots indicate cellular shrinkage and nuclear granulation. Initial magnification: 200x

In parallel with the morphological changes, the biochemical features of apoptosis developed. The degradation of nuclear DNA was detected by flow cytometry

with PI staining. Cells with a subdiploid DNA content were regarded as an apoptotic population. While the subG1 position of the vehicle-treated cells was <5%, the **3**, **4** or **7**-treated cells contained lower amounts of DNA, which increased concentration-dependently. At the highest concentration, the content of subG1 cells was about 20% (**Fig. 5**).

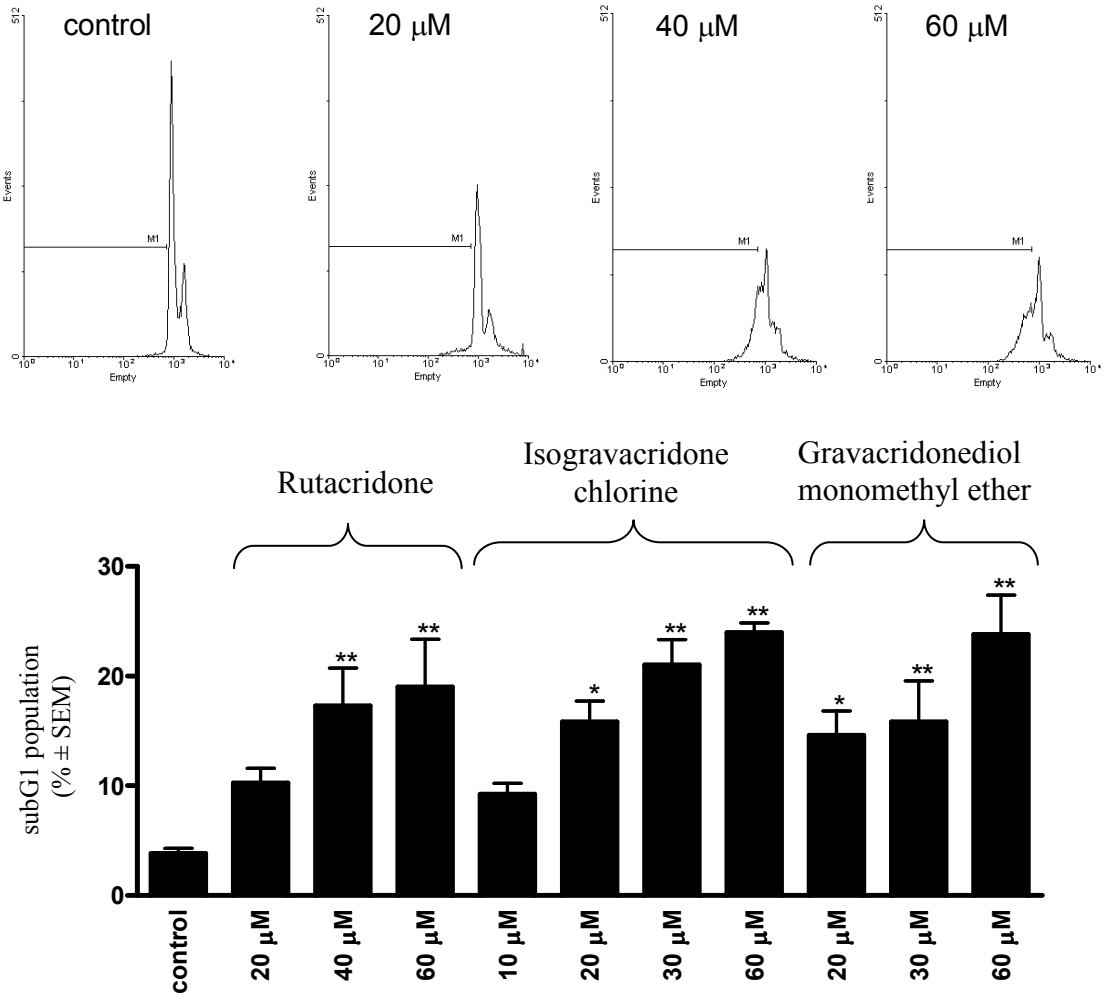


Figure 5. DNA content analysis: DNA fragmentation assay. Upper panel represents rutacridone-treated HeLa cells after 24 h. Lower panel: subG1 population of HeLa cells after 24-h treatment with isogravacridone chlorine, rutacridone or gravacridonediol monomethyl ether. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as compared with the control value.

To further characterize acridone-induced apoptosis, apoptosis-promoting (Bax) and apoptosis-preventing (Bcl-2) proteins were measured at the mRNA level using RT-PCR analysis. A concentration-dependent decrease in Bcl-2 mRNA expression was observed in HeLa cells treated with acridone alkaloids for 24 h. The decline in Bcl-2 was significant with both compounds. On the other hand, a significant effect was not observed on the Bax mRNA level, though there was a tendency. The increase in the

quotient Bax/Bcl-2 may explain the apoptotic effects of **3** and **4** (**Fig. 6**), which are partly mediated via a Bax/Bcl-2-regulated mitochondrial pathway.

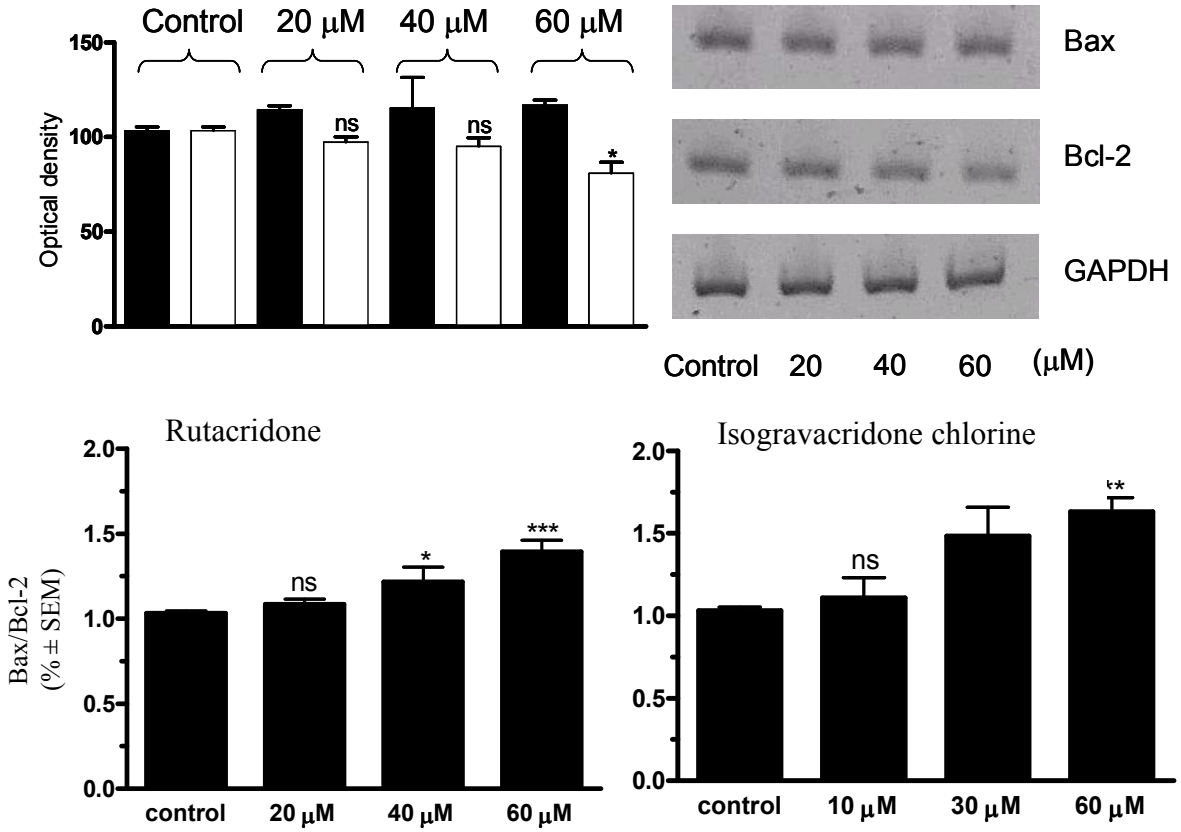


Figure 6. Upper panel: Effects of rutacridone on Bcl-2 and Bax mRNA expression. A representative figure for rutacridone-treated HeLa cells. Concentration-dependent decrease in Bcl-2 mRNA level at 24 h (black colums: Bax; white columns: Bcl-2). Lower panel: Effects of rutacridone and isogravacridone chlorine on the quotient Bax/Bcl-2 for HeLa cells after a 24-h treatment. *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, as compared with the control value**.**

4.2.2. Inhibition of MDR by acridone alkaloids

To evaluate the ability to inhibit Pgp-mediated drug efflux, acridone alkaloids were tested in the Rh-123 accumulation assay with the L5178 MDR mouse lymphoma cell line. The intracellular Rh-123 was measured by flow cytometry after a 30-min incubation period. At 40 μM, **3**, **4**, **5** and **6** all inhibited the pump function of Pgp more efficiently than the positive control verapamil (40.6 μM). Compounds **1** and **2** did not exert any effect on the drug accumulation at 40 μM, whereas at 400 μM all of the tested acridones displayed increases in FAR (**Table 5**). Of these compounds, **5** proved to have the most marked MDR reversal effect. At 400 μM, **5** increased the Rh-123

accumulation in MDR1 gene-transfected mouse lymphoma cells as compared with the sensitive cell line (**Fig. 7**).

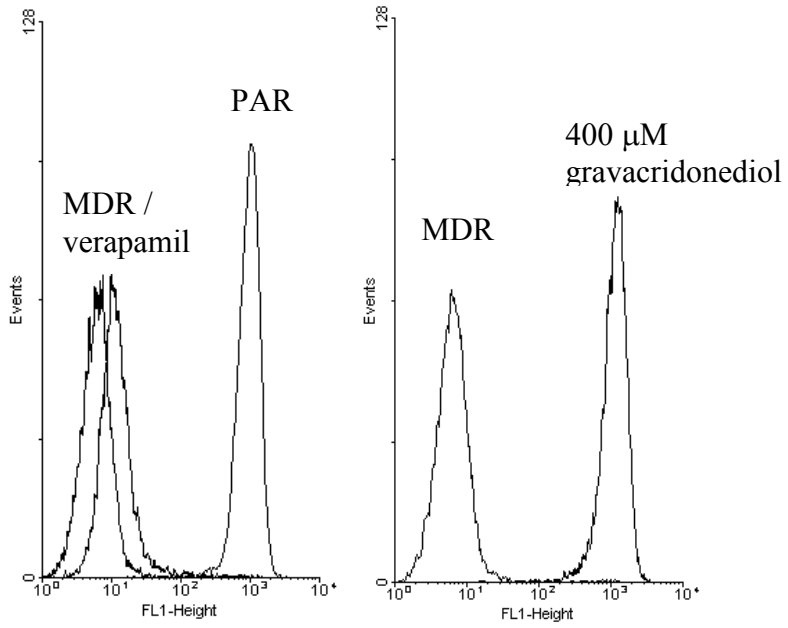


Figure 7. Effect of gravacridonediol on intracellular Rh-123 accumulation in L5178 MDR cells. Cells were treated with gravacridonediol for 30 min and assayed for Rh-123 accumulation. Left panel: A representative histogram of non-treated parent (PAR) and MDR cells and 40.6 μM verapamil-treated L5178 MDR cells. Right panel: Effects of 400 μM gravacridonediol on intracellular Rh-123 accumulation.

Table 5. Effects of acridones on Rh-123 accumulation assay in L5178 MDR mouse lymphoma cells.

Compound	μM	FAR	Compound	μM	FAR
1	40	1.72 ± 0.16	**5**	40	105.10 ± 16.34
	400	28.02 ± 4.08		400	130.30 ± 20.79
2	40	1.76 ± 0.47	**6**	40	16.11 ± 7.80
	400	20.17 ± 4.80		400	20.84 ± 8.18
3	40	18.69 ± 1.53	**7**	40	2.54 ± 0.12
	400	20.89 ± 3.34		400	43.77 ± 9.71
4	40	17.93 ± 2.77	Verapamil	40.6	2.18 ± 0.40
	400	10.96 ± 2.21	DMSO	4%	0.94 ± 0.07

Each result is the mean ± SEM of data from three experiments.

The antiproliferative effects of the tested acridones on Pgp expressed by L5178 murine cells are listed in **Table 6**. The sequence of antiproliferative potency was: **1** < **6** < **7** < **5** < **2** < **4** < **3**; compound **3** had the lowest IC_{50} value, which was comparable with that of doxorubicin (IC_{50} = 1.097 μM).

To assess the effects of the acridone alkaloids on doxorubicin-induced toxicity, MTT colorimetric assays were performed to determine the interactions between the acridones and doxorubicin, using the checkerboard method. Compounds **6** and **7** were able to enhance the antiproliferative activity of doxorubicin on the L5178 MDR cell line; synergism was detected for these two compounds. Although **5** was the most effective agent in the MDR reversal test, surprisingly the combination resulted in an additive antiproliferative effect. Additive interactions were also observed for the tricyclic acridones **1** and **2**. Compounds **3** and **4** exerted antagonism with doxorubicin.

Table 6. Calculated IC_{50} values and the results with doxorubicin combinations.

Compound	IC_{50} (μM)	FIX value	Interaction with doxorubicin
1	69.57	0.85	addition
2	33.22	0.71	addition
3	0.062	37.29	antagonism
4	16.02	2.19	antagonism
5	33.97	0.76	addition
6	67.21	0.41	synergism
7	43.65	0.03	synergism

Compounds **6** and **7** enhanced the cytotoxicity of doxorubicin in multidrug-resistant L5178 cells. In further tests, we examined the effects of these compounds in a non-cytostatic concentration (15 μM) on the MDR1 mRNA expression after a 48-h incubation.

RT-PCR studies showed that the MDR1 mRNA level was decreased after a 48-h treatment with compound **6** or **7** in L5178 MDR (**Fig. 8**).

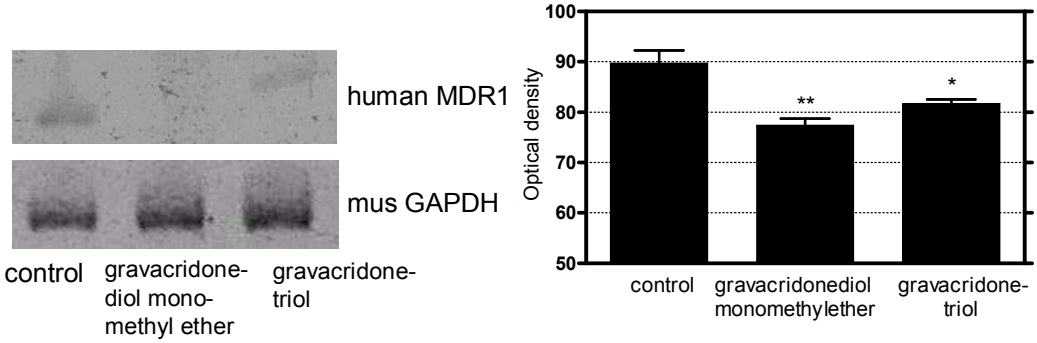


Figure 8. RT-PCR analysis of MDR1 mRNA expression. Effects of 48-h treatment with 15 μM gravacridonediol monomethyl ether or 15 μM gravacridonetriol on MDR1 mRNA expression in L5178 MDR cell line. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as compared with the control value.

5. DISCUSSION

Natural products provide one of the most important sources of promising leads for the development of novel chemotherapeutics in the areas of infectious diseases and cancer. Cragg and colleagues showed that, for the period 1989 to 1995, over 60% of the approved drugs developed for the treatment of cancerous and infectious diseases were of natural origin, including biological, natural and natural derived products [17,101]. Natural products exhibit a great variety of chemical structures, which underlines the important role of secondary metabolites as an important source for new leads.

5.1. Asteraceae screening

One of the most fruitful strategies for the discovery of natural products with biological activities is the performance of large-scale screening programmes utilizing high-throughput techniques. A colorimetric microplate-based assay utilizing the metabolic reduction of MTT was recently introduced in our department for evaluation of the cytotoxic effects of plant extracts on different cancer cells.

In the first step of our screening programme, four tribes of the Asteraceae family were chosen from the Hungarian flora. Of the 228 plant extracts representing 25 species, 25 exhibited >50% growth inhibitory activity on one or more of the cell lines. These 25 extracts were selected for further investigation of their anticancer activity, including the determination of IC_{50} values and measurement of the direct cytotoxic effects. The differences were calculated between the antiproliferative and cytotoxic assay results: a limited difference indicated that the extract has no selectivity for growing cells, while a high difference denoted a real antiproliferative effect without directly killing the exposed cells. Accordingly, extracts with this kind of anticancer profile are suggested to be worthy of further investigations, including bioassay-guided identification of the compounds responsible for activity.

The National Cancer Institute Guidelines specify that extracts with IC_{50} values <20 μg/ml can be regarded as active [102]. When the present results are considered from this aspect, it may be speculated that many of the tested Asteraceae species are promising sources of new natural products with cytostatic properties.

The measured activities were compared with the ethnomedicinal uses of the plants with potent antiproliferative activity (inhibition of cell proliferation >50% at 10 μg/ml) reported by Hartwell, and it was concluded that the present screening results for

E. canadensis, *E. annuus*, *A. artemisiifolia*, *H. annuus* and *X. italicum* are in accord with the traditional use of the plants against tumours and cancers [18]. Especially the root extracts (A and/or B) of *E. canadensis*, *E. annuus* and *H. annuus*, the root and leaf extracts (A and B) of *A. artemisiifolia,* and extracts (B) from all plant parts of *X. italicum* exhibit high activity against all of these cell lines. Some of the plants proved ineffective or exerted only a marginal effect on the cell lines used, in spite of their traditional use in cancer treatment. Thus, the extracts of *A. tinctoria* were found to be inactive, while the extracts of *A. campestris*, *A. dracunculus*, *A. vulgaris* and *T. inodorum* demonstrated a weak anticancer profile, although their ethnomedicinal use in European countries has been described [18]

A survey of the literature data on the species with substantial cytotoxic activity revealed that the antitumour activity of *E. canadensis*, *I. ensifolia* and *A. ruthenica* had not been reported earlier. Therefore, these species can be regarded as promising sources of new antitumour agents. In some cases, one or two cytotoxic constituents in the plants have been identified, but it can be presumed that the active compounds have not been completely exploited. For example, earlier reports on *A. asiatica* described only the cytostatic, apoptosis-inducing eupatilin [103, 104]. As concerns *A. japonica* ssp. *littoricola*, only dehydrofalcarindiol, with antitumour effects against colorectal and breast cancer, and eugenol, with melanoma growth-suppressive activity, have been described [105, 106]. A phytochemical study of the aerial parts of *A. artemisiifolia* resulted in the isolation of two cytotoxic sesquiterpenes, the pseudoguaianolide paulitin and isopaulitin, but the active compounds in the root extracts as yet remain unidentified [107]. These species are worthy of detailed phytochemical-pharmacological investigations in order to isolate further active compounds responsible for the antitumour activity. *E. annuus* is reported to contain gamma-pyranone derivatives and a sesquiterpene which possess cytotoxic activity [108, 109]. Cytotoxic triterpenoid saponins and sesquiterpene lactones have been isolated from *H. annuus* [110, 111]. Xanthanolides which presumably contribute to the outstandingly antiproliferative effect of the extract have been isolated from *X. italicum* [112].

Among the plants with high tumour cell growth-inhibitory activity, *I. ensifolia*, *A. collina*, *A. ruthenica* and *T. speciosa* have a rather limited distribution: they are native in several Central and Eastern European countries, indicating that our local flora can offer outstanding possibilities for the discovery of new structures with antitumour properties.

5.2. Identification of cytostatic compounds from the rhizome of *T. communis*

The bioassay-guided fractionation of the $CHCl_3$ fraction of the rhizome of *T. communis* led to the identification of 5 phenanthrenes. The cytotoxic assay of the isolated molecules revealed that compounds **10**-**12** and **14** exert marked cell growth-inhibitory activity on the HeLa cell line. We concluded that 2,7-dihydroxy-3,4,8-trimethoxy-phenanthrene (**12**) is worthy of interest because of its high activity (IC_{50} = 0.97 μM), which was comparable with those of the positive controls, cisplatin and doxorubicin (IC_{50} = 0.15 and 12.43 μM, respectively), while compound **13** exhibited no cytotoxic effect on HeLa cells. It is also noteworthy that one of the compounds (**10**) is a new natural product, while three of them (**11-13**) were described previously in other species.

A large variety of combretastatin analogues have been investigated in order to elucidate the structure-activity relationship. It is generally accepted that a diaryl system linked by a carbon-carbon double bond is essential for the cytostatic effect, together with the three methoxy groups on one of the rings [113-115]. Compounds isolated from *T. communis* can be regarded as conformationally restricted analogues of stilbenes. However, the two most active members of the currently tested phenanthrenes, compounds **12** and **14**, have only two methoxy groups on one of the rings. Moreover, the substance closest to combretastatin as regards the three methoxy groups, compound **10**, has only a limited cytostatic effect. These data do not fit into the previously published structure-activity relationship as concerns combretastain analogues. On the other hand, the olefinic part has been considered to be an additional target for modification of the chemical structure. Many conformationally restricted analogues, including sulfonate and azetidinone derivatives of combretastatin, have clearly revealed that inhibition of the rotation favours antiproliferative action [116, 117]. Accordingly, it is conceivable that the structure-activity relationship observed for the substituted stilbenes is not directly applicable to the phenanthrene skeleton.

5.3. Acridone alkaloids

Acridone alkaloids are secondary metabolites exclusively characteristic of the Rutaceae family; they have been isolated from many species. Natural acridones can be

divided into two major groups: tricyclic and tetracyclic compounds. Most of the members of the tetracyclic group carry an extra pyran ring in an angular position on the acridone nucleus; the furanoacridone skeleton is rather uncommon [118]. Such alkaloids have been found to date only in *R. graveolens* and *Thamnosma rhodesica*, an African woody herb [41]. Acronycine, a pyranoacridone, and glyfoline, a tricyclic acridone, isolated from *Acronychia baueri* and *Glycosmis citrifolia*, respectively (both Rutaceae), are well-characterized lead compounds for the development of anticancer agents [119]. Many congeners of both lead substances have been synthetized in order to describe the structure-activity relationship and therefore to improve the biological activity [120]. In spite of the intensive development of these synthetic analogues, pharmacological descriptions of the naturally occurring furan-condensed acridones are sparse and insufficient. Many activities have been published for **1** and **2**, but no evidence of their anticancer effect has been documented [121-123].

5.3.1. Cytostatic activity of acridones

In the set of experiments described here, we demonstrated that acridones from *R. graveolens* inhibit proliferation and induce apoptosis of the HeLa cells. All of the tested substances, with the exception of compound **2**, with a linear fourth ring formed by a methylenedioxy group at position C-2 – C-3, and glycosides **8** and **9**, exhibited considerably low IC_{50} values on each of the cell lines used. The most potent agent, the tricyclic arborinine, has been reported to exhibit moderate cytotoxic activity in the brine shrimp lethality test and in the potato disk bioassay, but it has not been tested by means of the MTT assay on human cell lines [124].

The antiproliferative values displayed a relatively close correlation (coefficient >0.7) for two of the three cell lines; the lowest correlation coefficient was slightly below 0.6. This clear parallelism allows the suggestion that the membrane permeability plays a crucial role in the cytostatic mechanism of these alkaloids and presumably involves the crossing of the cellular membranes. Although the precise mechanisms of action of the tested alkaloids have not been clarified, they presumably exert their cytostatic effects by a common mechanism. The further studies were therefore carried out only with some representatives in order to elicidate this mechanism.

Cell proliferation is defined as the increase in cell number resulting from completion of the cell cycle. The cell cycle is composed of four phases: the gap before

DNA replication (G1), the DNA synthetic phase (S), the interval after DNA replication (G2), and the mitotic phase (M) [125, 126]. As the cellular DNA content changes characteristically during these phases, a DNA-binding fluorescence probe makes it possible to determinate the distribution of a cell population.

The cell cycle analysis revealed that its distribution changes in acridone-treated HeLa cells. The proportion of the S phase increased, while the G2/M phase decreased, leading to an increase in the quotient S/(G2/M). The change in this parameter was significant in the cases of compounds **3** and **7**, while for compound **4** it resulted only in a trend. These data suggested a perturbation of the cell cycle, blocking the transition from the S to the G2 phase. Similar results were earlier found for derivatives of acronicyne, which could indicate a common mechanism for these acridones [118].

The apoptosis-induction capacity rather than necrosis induction is accepted as a key feature of a potential antitumour drug. Accordingly, in the next set of experiments, the apoptotic potentials of the tested agents were investigated.

Staining with EB and AO is sufficient for a qualitative documentation of apoptosis. Typical morphological markers, including cellular shrinkage, nuclear condensation and increased membrane permeability, were observed after a 24-h treatment with 3 furanoacridones. For a quantitative characterization, the same compounds were subjected to flow cytometric analysis of the DNA content. It was observed that all of the tested compounds concentration-dependently increased the subdiploid DNA (subG1) population of the HeLa cells, and the highest proportion of apoptotic cells was approximately 25%. The characteristic structural appearance and the augmentation of the apoptotic subG1 population indicate that apoptosis is a major underlying process induced by acridones.

The Bcl-2 family members appear to interact to regulate the commitment to survive or die upon challenge with various apoptotic stimuli by controlling the flux of ions and proteins through intracellular membranes [71]. Bcl-2 and Bcl-x_L have been shown to exert their inhibitory effects on apoptosis by blocking the release of cytochrome *c* and the decline in mitochondrial membrane potential ($\Delta\Psi_m$). Bax mainly resides in the cytosol and translocates to the mitochondria upon receiving an apoptotic signal, where it may initiate homodimerization or heterodimerization, resulting in a mitochondrial dysfunction. The quotient Bax/Bcl-2 is important for the survival of drug-induced apoptosis [127]. Here we have demonstrated that treatment with rutacridone or isogravacridone chlorine results in a concentration-dependent increase in

the quotient Bax/Bcl-2 at the RNA expression level, indicating that these substances can shift the anti-apoptotic – pro-apoptotic balance of the cell towards undergoing apoptosis. This provides evidence that an increase in the quotient Bax/Bcl-2 is another mechanism of apoptosis of HeLa cells induced by acridones.

5.3.2. Reversal of MDR

MDR is one of the greatest challenges in chemotherapy, limiting the efficacy of numerous anticancer agents. The intracellular levels of many chemotherapeutic drugs are decreased by the activity of ABC proteins such as Pgp, which are upregulated in drug-resistant cancer cells. The development of agents which inhibit the Pgp-mediated efflux of drugs and thus reverse MDR has therefore been intensively pursued. Many substances have been found that are promising in inhibiting Pgp *in vitro.* Clinical trials have been disappointing, however, due either to dose-limiting toxicity or to unpredictable drug interactions. Non-toxic Pgp inhibitors derived from plants may prove to be efficacious when administered in combination with commonly used chemotherapeutic drug such as doxorubicin.

A number of naturally occurring and synthetized acridones were recently tested in drug accumulation assays [128, 129]. One of the most extensively examined agents is GF120918, an acridonecarboxamide derivative, which displays potent Pgp inhibition activity [130]. In the present project, we examined the effects of some acridones isolated from *R. graveolens* on the Pgp-mediated MDR of drug-resistant cells.

All of the tested compounds enhanced the intracellular Rh-123 accumulation in drug-resistant L5178 mouse lymphoma cells. The most effective compound was gravacridonediol, which resulted in a similar extent of drug accumulation in the drug-sensitive PAR cell line. The sequence of antiproliferative potency was **3** > **4** > **2** > **5** > **7** > **6** > **1**. A comparison of the cytostatic results for the furacridones (**3**-**7**) on mouse lymphoma cells with our previous results on the HeLa, MCF7 and A431 cell lines revealed that gravacridonetriol was always less active than the other furacridones, and the IC_{50} values of isogravacridone chlorine and rutacridone were in the first places in the sequence.

In the combination study, some compounds were more effective than expected from the accumulation assay. This method is frequently used to detect interactions between antimicrobial agents, but can be applied in cytotoxic studies too [131]. The

combination of doxorubicin and compound **5** resulted only in addition, but in the cases of compounds **6** and **7** there was a synergistic effect. Since the duration of exposure in the Rh-123 accumulation test was short (30 min), it is unlikely that acridones act by down-regulating MDR1 transcription and, hence, only the direct inhibition of the pump can be responsible for the rhodamine accumulation. Compounds **6** and **7** were chosen to investigate the effects of the acridones on the expression of MDR1 on mRNA levels after a longer exposure. A significantly decreased level of gene expression was detected, indicating that these acridones increase intracellular drug levels by modulating Pgp activity and the expression of MDR1.

6. CONCLUSIONS

In this work, naturally occurring compounds with cytostatic activity towards tumour cells were sought, detected and evaluated. Screening of 4 extracts of various plant parts of the Asteraceae family for antiproliferative activity resulted in promising starting plants for further investigation. Extracts which exhibited substantial antiproliferative activity may represent a source for novel natural anticancer entities.

Bioassay-guided fractionation of *T. communis* extracts led to the purification of a set of highly potent antiproliferative phenanthrenes, including 7-hydroxy-2,3,4-trimethoxyphenanthrene, a new natural compund.

The effects of acridone alkaloids on cell proliferation and apoptosis may involve different mechanisms, and we have demonstrated here that acridones change the cell cycle and regulate the mRNA expression of Bax and Bcl-2. The molecular mechanism that regulates the balance between cell proliferation and cell apoptosis needs further investigations. The present results indicate that some of the acridones are worthy of further study, including *in vivo* tumour models.

Furanoacridones proved to be effective in reversing Pgp-mediated MDR in the Rh-123 accumulation assay. This effect was reinforced by a potentiation of the cytostatic effect of doxorubicin by some of the acridones.

In summary, our results indicate that naturally occurring plant components, including acridone alkaloids and phenanthrenes, may be used as starting structures for the potential development of novel anticancer agents.

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