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

PhD thesis

Borbála Réthy

Szeged
2007

Department of Pharmacodynamics and Biopharmacy,
Faculty of Pharmacy, University of Szeged

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

PhD thesis

Borbála Réthy

Supervisor:
Prof. George Falkay Ph.D., D.Sc.

Szeged
2007

INTRODUCTION

Cancer is one of the most prevalent diseases in many countries worldwide. Cancer is a term describing conditions characterized by uncontrolled cellular proliferation and dedifferentiation. The exact cause of the disease remains enigmatic, but inhibition of the defence mechanism responsible for the elimination of disturbed cells is generally accepted as a background of carcinogenesis.

Apoptosis, or programmed cell death, is one of the most finely coordinated regulatory functions for maintenance of the homeostasis in the living organism. It involves the continuous checking of the cellular integrity and a cascade-like events of self-destruction when the integrity of the organism is endangered. Morphological hallmarks of apoptosis are nuclear condensation, cell shrinkage, membrane blebbing and the formation of apoptotic bodies. These changes are accompanied by biochemical features, including DNA fragmentation and the proteolytic cleavage of a variety of intracellular substrates. Caspases are the best-characterized enzymes that perform this, while the Bcl-2 family is the principal set of proteins that regulate the apoptotic cascade.

The multidrug-resistance (MDR) of tumour cells to chemotherapeutic agents is a major problem in the clinical treatment of cancer. MDR is defined as the ability of malignant cells exposed to chemotherapeutics to develop resistance to a broad range of drugs due to the members of the ATP-binding cassette proteins. The best-examined member of the family is P-glycoprotein (Pgp), identified first in Chinese hamster ovary cells by Juliano and Ling in 1976.

The search for novel anticancer agents currently targets chemical entities that selectively induce apoptosis and/or reverse MDR. Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural sources. Plant extracts can be regarded as chemical libraries of structurally diverse compounds, their investigation therefore constituting a promising approach in drug discovery.

AIMS

In accordance with the preceding approach, our aims were as follows:

- To select plants, partly on the basis of literature surveys, for antiproliferative investigation. Hungarian species of the Asteraceae family were subjected to a systematic screening for cytostatic activity.
- To identify pure compounds responsible for the detected cytotoxic activity of the extracts of *Tamus communis* L.
- To investigate previously isolated, but not tested acridones from *Ruta graveolens* L. for anticancer activity. We focused on the elucidation of the mechanism of their apoptosis-inducing effects.
- To explore acridones with the ability to inhibit Pgp-mediated MDR and to clarify their potential importance in a set of drug combination experiments.

MATERIALS AND METHODS

Plant material

The initial set of Asteraceae plants were collected in the flowering period from several regions of Hungary. Four crude extracts were prepared with *n*-hexane (extract A), chloroform ($CHCl_3$) (extract B), and water (extract C). The residual plant material was extracted with boiling water, and freeze-dried (extract D).

In the case of *T. communis*, the rhizomes were collected in the Mecsek Hills. The effective agent was determined by bioassay-guided fractionation, and finally identified.

The acridone alkaloids were provided 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**)).

Tumour cell lines

Human (MCF7, HeLa and A431) and mouse (L5178 T-cell lymphoma, sensitive and Pgp-expressing variants) cancer cell lines were used. All these cell lines were cultured in the appropriate medium in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay

Antiproliferative effects were measured *in vitro* by using MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assays. After treatment, 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. 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). Stock solutions of the tested materials 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 (dose-response curve and cytotoxicity).

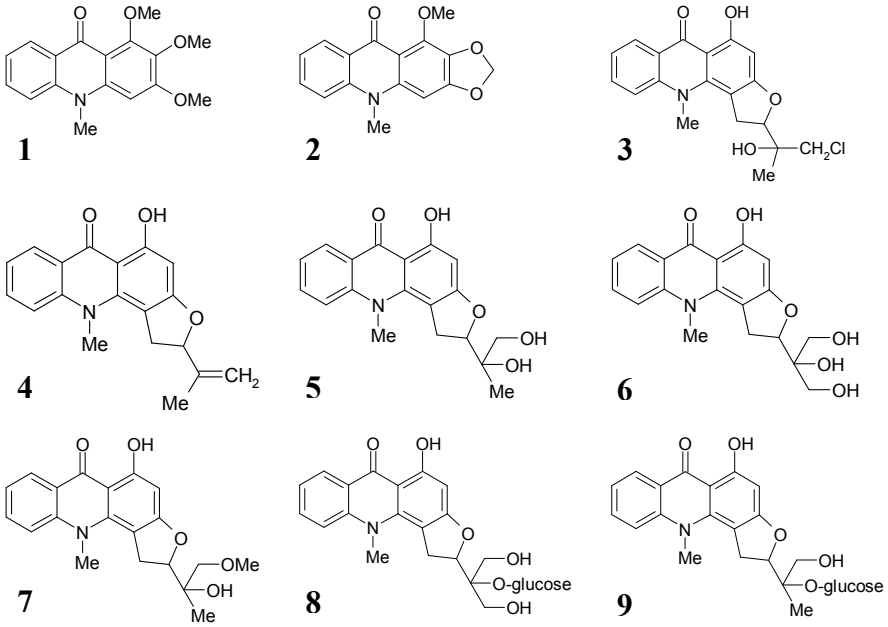
To study the interactions between acridones and doxorubicin, a checkerboard method was applied. A series of 2-fold dilutions of the acridones was tested in combination with 2-fold dilutions of doxorubicin. The cell growth rate was determined with MTT staining; drug interactions were evaluated according to the following system (fractional inhibitory index = FIX):

FIX < 0.5	Synergism	1 < FIX < 2	Indifferent effect
FIX = 0.51-1	Additive effect	FIX > 2	Antagonism

Transmembrane permeability assay

The transmembrane permeabilities were determined in parallel artificial membrane permeability assays (PAMPA) and log P_e values were calculated. *n*-Dodecane in *n*-hexane was used as lipid bilayer 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.

Figure 1. Chemical structures of the acridone alkaloids.



Ethidium bromide and acridine orage dual staining

Staining with ethidium bromide (EB) and acridine orange (AO) was carried out after 24-h acridone treatment to visualize the basic morphological events of apoptosis. This staining allowed the identification of intact, early-apoptotic, late-apoptotic and necrotic cells. AO permeates all cells and makes the nuclei appear 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.

Flow cytometric analysis

For the measurement of cellular DNA content stained with propidium iodide, flow cytometric analysis was used after a 24-h treatment of HeLa cells. The percentages of the cells in the different cell-cycle phases (G1, S and G2/M) were calculated; the subdiploid fraction was regarded as the apoptotic cell population.

The retention of rhodamine 123, an indicator of the total cellular efflux activity, was detected by flow cytometry as a functional index of Pgp activity. Verapamil was used as a positive control. The fluorescence activity ratio was calculated as the ratio of the fluorescence of the treated MDR and untreated cells.

Reverse transcriptase-PCR studies

The effects of the tested compounds on the mRNA expression pattern of Bax, Bcl-2 and MDR1 were determined by RT-PCR technique. After treatment (24 h for HeLa, 48 h for L5178 MDR cells), 10^6 cells were denaturated and cDNA was prepared in the presence of RT. Human and mouse glyceraldehyde-3-phosphate dehydrogenase primers were used as internal control. The sequences of the oligonucleotide primers for Bcl-2, Bax and MDR1 were the same as previously reported.

RESULTS

Asteraceae screening

Species from the Asteraceae family were selected for screening of their antiproliferative effect, 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), Helianthae (5) and Anthemideae (11)], 228 different extracts were prepared from selected plant organs. These extracts were initially screened on three human cancer cell lines at 10 μg/ml. This first selection resulted in 25 extracts that exerted ≥50% inhibition of proliferation on one or other cell line. For the selected extracts, IC_{50} values were determined; direct cytotoxic and cytostatic effects were distinguished at 30 μg/ml.

Of 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 (fraction A and C, respectively) were also effective. None of the residual aqueous extracts (fraction D) demonstrated a pronounced antiproliferative effect (>50% inhibition) against any cell line.

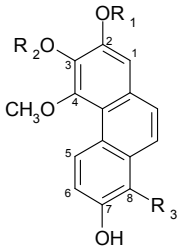
For the active Anthemideae species, the extracts of the aerial parts proved to be effective. The extracts of *A. collina* exhibited some selectivity for dividing cells, as evidenced by the high cytostatic-cytotoxic differences. The extract from the roots of the Astereae plants seemed to be more effective than those from other organs, and the MCF7 cells were slightly more sensitive than the other two cell lines. The *n*-hexane extracts of the roots of *E. canadensis* exhibited the highest activity in the tribe, and displayed substantial direct cytotoxic action. In the Heliantheae tribe, noteworthy antiproliferative activities were recorded for *A. artemisiifolia*, *H. annuus* and *X. italicum.* 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. As concerns the Inuleae species, *I. ensifolia* was found to be moderately active, while *T. speciosa* proved to have marked efficacy.

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 fractionation on HeLa cells, using the MTT assay. In the first set of experiments, the $CHCl_3$ extract exhibited pronounced antiproliferative activity (IC_{50} 0.02 μg/ml), while the MeOH extract was inactive. For identification of components responsible for the effect of the extract, it was fractionated into 13 fractions. The fractions IV, V+VI, VIII, IX and XIII exhibited >50% activity at a concentration of 10 μg/ml. In further appropriate chromatographic steps, 5 active phenanthrenes were isolated from these active fractions.

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 (**Table 1**). Compound **10** is a new natural compound, while **11** (nudol), **12** (confusarin) and **13** were first described in *T. communis*. Compound **14** (previously reported as TaVIII) was earlier isolated from the rhizomes of this plant.

Table 1. The structures and cytostatic activities of the phenanthrenes isolated from *T. communis* on HeLa cells.



	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

Antitumour effects of the acridones

The antiproliferative effects and the lipid solubilities of the investigated compounds are listed in **Table 2.** Compound **1** proved to be an outstandingly potent cytostatic agent, especially against HeLa cells. The tested furacridones (**3-9**) exhibited slightly different sequences of cytostatic potency. The permeabilities of these compounds were characterized by PAMPA and log P_e values were calculated. The correlation coefficients between log P_e and IC_{50} were found to be 0.717, 0.856 and 0.578 for HeLa, MCF-7 and A431 cells, respectively.

Table 2. IC_{50} and log P_e values of the tested compounds. * indicates that the compound did not inhibit cell proliferation to a significant level at 30 mM.

Compound	IC_{50} values (μM)			log P_e values
	HeLa	MCF7	A431	
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.

n.d.: not determined

The effect on the cell cycle profile was characterized by measuring the cellular DNA content. In the further tests, three selected acridones were studied for elucidation of their mechanism of action. The flow cytometry revealed that **3**, **4** and **7** exerted effects on the cell cycle distribution. A 24-h exposure of HeLa cells resulted in an increased proportion of cells in the S phase, and a decreased proportion of cells in the G_2/M phase. (**Fig. 2**).

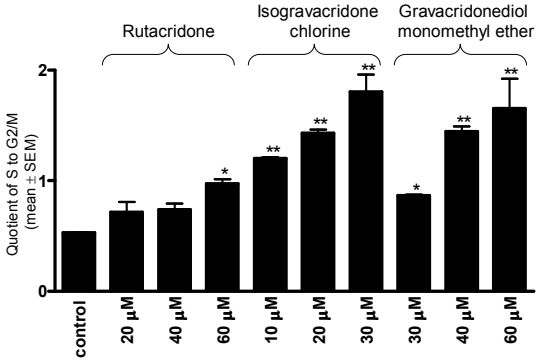


Figure 2. Cell cycle analysis: effects of acridones on HeLa cells after 24-h treatment. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as compared with the control value.

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 degradation of nuclear DNA was detected by flow cytometry While the subG1 position of the vehicle-treated cells was <5%, **3**, **4** and **7**-treated cells cantained a lower amount of DNA, which increased concentration-dependently (**Fig. 3**).

To further characterize acridone-induced apoptosis, apoptosis-promoting (Bax) and apoptosis-preventing (Bcl-2) proteins were measured at the mRNA level. A concentration-dependent decrease in Bcl-2 mRNA expression was observed in HeLa cells treated with acridone alkaloids for 24 h. On the other hand, only a tendency to an increase was observed at the Bax mRNA level. The increase in Bax/Bcl-2 may explain the apoptotic

effects of **3** and **4** (**Fig. 4**), which is partly mediated via a Bax/Bcl-2-regulated mitochondrial pathway

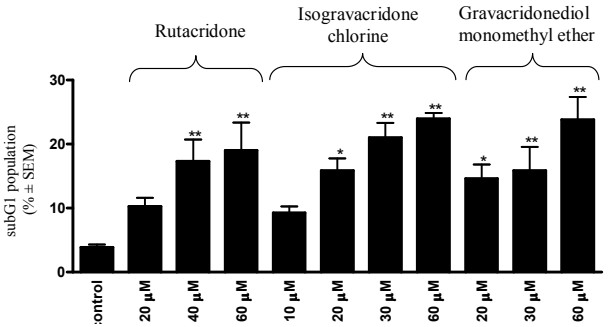


Figure 3. DNA fragmentation assay. subG1 population of HeLa cells after 24-h treatment with acridones. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as compared with the control.

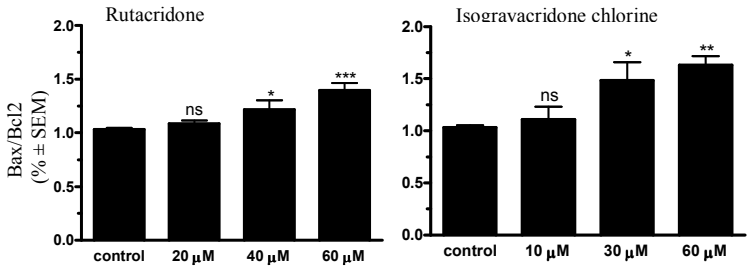


Figure 4. Effects of rutacridone and isogravacridone chlorine on Bax/Bcl-2 mRNA expression of HeLa cells after a 24-h treatment *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, as compared with the control value**.**

Inhibition of MDR by acridone alkaloids

To evaluate the ability to inhibit the Pgp-mediated drug efflux, acridone alkaloids were tested in the rhodamine 123 accumulation assay on the L5178 MDR mouse lymphoma cell line. 40 μM of compound **3**, **4**, **5** or **6** inhibited the pump function of Pgp more efficiently than the positive control verapamil at 40.6 μM. Of these compounds, **5** proved to have the most marked MDR reversal effect (**Table 3**).

Compound	IC_{50} (μM)	Interaction with doxorubicin
1	69.57	addition
2	33.22	addition
3	0.062	antagonism
4	16.02	antagonism
5	33.97	addition
6	67.21	synergism
7	43.65	synergism

Table 3. Effect of acridones on the Rh-123 accumulation assay in L5178 MDR cells.

DISCUSSION

In this work, naturally occurring compounds with cytostatic activity towards tumour cells were sought, detected and evaluated. Screening of four extracts of plants of the Asteraceae family for antiproliferative activity resulted in promising starting sources for further investigation. The measured activities were compared with the ethnomedicinal uses of the plants with potent antiproliferative activity 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. A survey of the literature data revealed that the antitumour activities of *E. canadensis*, *I. ensifolia* and *A. ruthenica* had not been reported earlier. Extracts which exhibited substantial antiproliferative activity may represent a source for novel natural anticancer entities.

The bioassay-guided fractionation of the $CHCl_3$ fraction of the rhizomes from *T. communis* led to the identification of five phenanthrenes. The cytotoxic assay of the isolated molecules revealed that compounds **10**-**12** and **14** exerted marked cell growth-inhibitory activity on the HeLa cell line. Compound **10** is a new natural product, while **11**-**13** had been previously described in other species.

The effects of acridone alkaloids on cell proliferation and apoptosis may involve different mechanisms. Compound **3** and **7** increased the S phase, while G2/M was decreased, suggesting a perturbation of the cell cycle, blocking the transition from the S to the G2 phase. Cellular shrinkage, nuclear condensation and increased membrane permeability (typical morphological apoptotic markers) were observed after the 24-h treatment. The subdiploid DNA population and the increased Bax/Bcl-2 mRNA confirmed the apoptosis-inducing activity of acridones.

Furanoacridones proved to be effective in reversing Pgp-mediated MDR in the rhodamine 123 accumulation assay. This effect was reinforced by a potentiation of the cytostatic effect of doxorubicin by some of the acridones. Compounds **6** and **7** not only blocked the function of Pgp, but also decreased the mRNA expression of MDR1.

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.

ANNEX

Publications directly related to the subject of the dissertation:

I. **Réthy B**, Kovács A, Zupkó I, Forgo P, Vasas A, Falkay G, Hohmann J. Cytotoxic phenanthrenes from the rhizomes of *Tamus communis*. Planta Med 2006;72:767-70.
IF_{2006}: 1.746

II. **Réthy B**, Zupkó I, Minorics R, Hohmann J, Ocsovszki I, Falkay G. Investigation of cytotoxic activity on human cancer cell lines of arborinine and furanoacridones isolated from *Ruta graveolens*. Planta Med 2007;73:41-8.
IF_{2006}: 1.746

III. **Réthy B**, Csupor-Löffler B, Zupkó I, Hajdú Z, Máthé I, Hohmann J, Rédei T, Falkay G. Antiproliferative activity of Hungarian Asteraceae species against human cancer cell lines. Part I. Phytother Res (accepted for publication.)
IF_{2006}: 1.144

Publications indirectly related to the subject of the dissertation:

IV. Kovács A, Forgo P, Zupkó I, **Réthy B**, Falkay G, Szabó P, Hohmann J. Phenanthrenes and a dihydrophenanthrene from *Tamus communis* and their cytotoxic activity. Phytochemistry 2007;68:687-91.

V. Csomós P, Zupkó I, **Réthy B**, Fodor L, Falkay G, Bernáth G. Isobrassinin and its analogues: Novel types of antiproliferative agents. Bioorg Med Chem Lett 2006;16:6273-76.

Lectures and poster presentations:

1. **Réthy B**, Zupkó I, Falkay G. A patkány anyai agy noradrenerg és dopaminerg transzmissziójának vizsgálata szuperfúziós technikával. Ph.D. Tudományos Napok 2004. 2004 április 8-9, Budapest.

2. Forgo P, Hohmann J, Dombi G, Zupkó I, **Réthy B**, Falkay G. Isolation, structure determination and cytotoxic activity of Amaryllidaceae alkaloids. Second International Symposium on Chemistry, Biological and Pharmacological Properties of Medicinal Plants from the Americas. 15-18 August, 2004, São Pedro, Brazil.

3. **Réthy B.** Természetes és félszintetikus akridon-szrmazékok citotoxikus hatásának vizsgálata. VII. Clauder Ottó Emlékverseny. 2004. október 14-15, Visegrád.

4. **Réthy B**, Zupkó I, Hohmann J, Falkay G. A *Ruta graveolens*ből izolált citotoxikus anyagok vizsgálata in vitro. A Magyar Tudomány Ünnepe Szegeden. 2004. november 2-19, Szeged.

5. **Réthy B**, Kovács A, Zupkó I, Hohmann J, Falkay G. A *Tamus communis*-ból izolált fenantrének citotoxikus hatásának vizsgálata in vitro. Ph D Tudományos Napok 2005. Semmelweis Egyetem Doktori Iskola. 2005. április 14-15, Budapest.

6. **Réthy B**, Zupkó I, Hohmann J, Falkay G. Investigation of cytotoxic activity of naturally occurring acridone alkaloids. Joint Meeting on Medicinal Chemistry. 20-23 June, 2005, Vienna, Austria.

7. Hajdú Z, Csupor-Löffler B, Hohmann J, **Réthy B**, Zupkó I, Falkay G, Máthé I. In vitro cytotoxicity of extracts from Hungarian Asteraceae. 53rd Annual Meeting of the Society of Medicinal Plant Research. 21–25 August, 2005, Florence, Italy.

8. Vasas A, Rédei D, Kovács A, Forgó P, Zupkó I, **Réthy B**, Falkay G, Hohmann J. Cytotoxic phenanthrenes from the rhizomes of *Tamus communis* L. 53rd Annual Meeting of the Society of Medicinal Plant Research. 21–25 August, 2005, Florence, Italy

9. Csupor-Löffler B, Hajdú Z, **Réthy B**, Zupkó I, Forgó P, Hohmann J. A cickafarkfű citotoxikus anyagainak vizsgálata. XI. Magyar Gyógynövény Konferencia. 2005. október 13-15, Dobogókő.

10. Hajdú Z, Csupor-Löffler B, Hohmann J, **Réthy B**, Zupkó I, Falkay G, and Máthé I. Az Asteraceae család Magyarországon előforduló fajainak in vitro citotoxicitási vizsgálata. XI. Magyar Gyógynövény Konferencia. 2005. október 13-15, Dobogókő.

11. Kovács A, Vasas A, Rédei D, Forgó P, Zupkó I, **Réthy B**, Falkay Gy, Szabó LGy, Hohmann J. A pirítógyökér (*Tamus communis* L.) citotoxikus hatású anyagai. XI. Magyar Gyógynövény Konferencia. 2005. október 13-15, Dobogókő.

12. **Réthy B**, Zupkó I, Molnár J, Falkay G. Akridon-vázas alkaloidok tumourellenes hatásának vizsgálata in vitro. Ph. D. Tudományos Nap. Szegedi Tudományegyetem, MTA Szegedi Akadémiai Bizottság. 2006. május 3, Szeged.

13. Kovács A, Vasas A, **Réthy B**, Zupkó I, Forgó P, Hohmann J. Citotoxikus fenantrének izolálása a *Tamus communis* L.-ból. Congressus Pharmaceuticus Hungaricus XIII. 2006. május 25-27, Budapest.

14. **Réthy B**, Hohmann J, Ocsovszki I, Molnár J, Zupkó I, Falkay G. Akridon-alkaloidok multidrog rezisztencia módosító és apoptózist indukáló hatásának vizsgálata. Congressus Pharmaceuticus Hungaricus XIII. 2006. május 25-27, Budapest.

15. Zupkó I, **Réthy B**, Csupor-Löffler B, Hohmann J, Hajdú Z, Ocsovszki I, Falkay G. Magyarországi növényfajok citotoxikus hatású anyagainak vizsgálata. Congressus Pharmaceuticus Hungaricus XIII. 2006. május 25-27, Budapest.

16. **Réthy B**, Hohmann J, Ocsovszki I. Molnár J, Zupkó I, Falkay G. Apoptosis induction by acridone alkaloids and effect on reversal of multidrug resistance. 15th World Congress of Pharmacology. 2-7 July, 2006, Beijing, China.

17. **Réthy B**. Növényi eredetű anyagok tumourellenes hatásának vizsgálata. Magyar Tudomány Ünnepe Szegeden. 2006 november 9, Szeged.

18. Hajdú Z, Csupor-Löffler B, Hohmann J, **Réthy B**, Zupkó I, Falkay G, Máthé I. Magyarországon előforduló Asteraceae fajok *in vitro* antiproliferatív hatásának vizsgálata. Anniversary Scientific Session. 23-24 April, 2007, Targu-Mures, Romania.

19. **Réthy B**, Minorics R. A *Ruta graveolens*-ből izolált akridonok tumourellenes hatásának vizsgálata. VIII. Clauder Ottó Emlékverseny. 2007. április 12-13, Budapest.

20. Kovács A, Vasas A, Zupkó I, **Réthy B**, Forgo P, Hohmann J. Antitumor activity of xanthanolides from *Xanthium italicum* Moretti. 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research. 2-6 September, 2007, Graz, Austria.

21. Csupor-Löffler B, Hajdú Z, **Réthy B**, Zupkó I, Falkay G, Forgo P, Hohmann J. Activity-guided isolation of antiproliferative compounds from *Achillae collina*. 55th International Congress and Annual Meeting of the Society for Medicinal Plant Research. 2-6 September, 2007, Graz, Austria.