Isolation and Structure Elucidation of Compounds with Antitumor Activity from *Tamus communis* and *Xanthium italicum*

Summary of Ph.D. Thesis

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Introduction

Natural products play a highly significant role in the drug discovery and development process. They not only serve as drugs or templates for drugs directly, but in many instances lead to the discovery of novel mechanisms of action that provide a better understanding of the targets and pathways involved in the disease process. Natural products have additionally been used as starting templates in the synthesis of combinatorial libraries.

Recent progress towards the discovery of drugs from natural product sources has resulted in compounds that are being developed to treat different diseases, especially cancer, resistant bacteria and viruses and immunosuppressive disorders. In the area of cancer chemotherapy, some 67 % of the effective drugs may be traced to a natural origin (*e.g.* vinblastine, vincristine, paclitaxel, podophyllotoxin and camptothecin).

Currently one of the most interesting areas of antitumor drug research is the group of *cis*-stilbene combretastatins because of their potential use in cancer chemotherapy. The most potent member, combretastatin A-4, originally isolated from the southern African plant *Combretum caffrum*, in disodium phosphate prodrug form is undergoing testing in clinical trials, and has been found to be effective against different solid tumors, including multidrug-resistant cancers.

The alkoxy-substituted phenanthrenes, which are conformationally restricted congeners of the antitumor *cis*-stilbenes, have not been investigated in detail, but on the basis of their structural similarity they can be regarded as promising anticancer agents.

Sesquiterpenes are another group of secondary plant metabolites which have been investigated extensively for their antitumor effects. Some species of the genus *Xanthium* (Asteraceae) were used in traditional medicine for the treatment of basal cell carcinoma and different cancers and cold tumors, their extracts and sesquiterpene compounds exhibiting high activity against several human cell lines.

In recent years, Hohmann *et al*. initiated a research programme at the Department of Pharmacognosy, in collaboration with the Department of Pharmacodynamics and Biopharmacy, University of Szeged, with the aim of the isolation and identification of antitumor compounds from medicinal plants. As part of these studies, my

research activities have involved investigations of *Tamus communis* L. and *Xanthium italicum* Moretti. The present thesis summarizes the results of this phytochemical work.

Aims of the study

The aims of the present work related to the bioassay-guided isolation, identification and antitumor evaluation of compounds derived from *T. communis* and *X. italicum*. In order to achieve these aims, the main tasks were:

- The collection of the plant materials
- The screening of *T. communis* and *X. italicum* for tumor cell proliferation-inhibitory activity
- The extraction of the plant materials
- The bioassay-guided fractionation of the active extracts and purification of the compounds responsible for the activity by means of combinations of various chromatographic methods (OCC, VLC, CPC, preparative TLC, HPLC and gel chromatography)
- The characterization and structure determination of the isolated compounds via spectroscopic techniques (UV, NMR and HREIMS)
- The evaluation of the pharmacological potential of the isolated compounds.

Materials and methods

The antiproliferative activities of the extracts and the pure compounds were tested on different tumor cell lines through use of the MTT assay. The compounds were isolated in multistep separation procedures, including different extraction and chromatographic methods (OCC, VLC, CPC, preparative TLC, and NP- and RP-HPLC). The isolated compounds were characterized and their structures were elucidated by means of various spectroscopic methods (UV, MS and NMR).

Results and discussion

Bioassay-guided isolation of phenanthrenes and xanthanolides

The fresh rhizomes of *T. communis* were exhaustively percolated with MeOH at room temperature (Figure 1). The MeOH extract was concentrated and extracted with petroleum ether and $CHCl_3$. The organic fractions were tested for their cytotoxic activity on the HeLa cell line, using the MTT assay. Both the petroleum ether and $CHCl_3$ fractions exhibited high, concentration-dependent cytotoxic activity, and therefore both fractions were subjected to detailed phytochemical studies. They were chromatographed by vacuum liquid chromatography (VLC) on silica gel, in order to separate the constituent compounds according to their polarities.

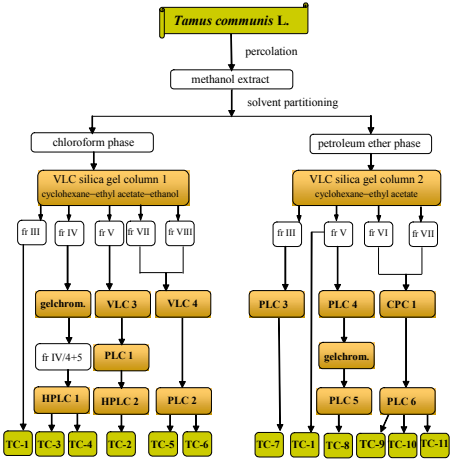


Figure 1. Isolation of phenanthrenes from *T. communis*

This chromatography resulted in a crude fractionation of the main components. The fractions were monitored by TLC and, after appropriate separation combinations, 12 main subfractions (I-XII) were obtained from the chloroform fraction, and 8 main subfractions (I-VIII) from the petroleum ether fraction and tested for their cytotoxic activity on the HeLa cell line (Figure 2). Subfractions IV, V, VIII and XII of the $CHCl_3$ phase, and subfractions V and VI of the petroleum ether phase were found to exert significant activity.

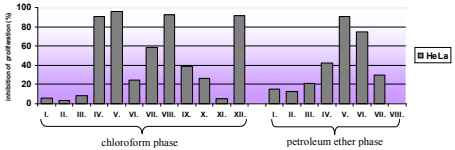


Figure 2. Antiproliferative activities of the VLC subfractions of *T. communis* on the HeLa cell line

Dried and powdered plant parts (root, flower, stem and leaf) of *X. italicum*, collected before and during the flowering period, were extracted with MeOH. After evaporation, the extracts were subjected to solvent–solvent partitioning, to yield *n*-hexane-, $CHCl_3$- and H_2O-soluble phases. These fractions, together with the original MeOH extract, were tested for their tumor cell proliferation-inhibitory activities on the HeLa, A431 and MCF7 cell lines. It was observed that the active components predominantly accumulated in the leaves and flowers, especially in the *n*-hexane and $CHCl_3$ extracts. In the next experiment, the dried leaves were extracted and the extract was partitioned between *n*-hexane, $CHCl_3$ and H_2O. Thereafter, a specific purification method was used (OCC on polyamide), with a gradient system of MeOH–H_2O, which resulted in a xanthanolide-rich fraction free from chlorophyll (Figure 3). Repeated VLC separations of the xanthanolide fraction afforded subfractions containing only a few main components. The fractions were combined according to their compositions, yielding subfractions I-V, of which subfractions I, II and IV exhibited pronounced cell growth-inhibitory activities (Figure 4). The subsequent purification methods were chosen with regard to the degree of purity and complexity of the fractions. Subfraction I was fractionated by VLC, This resulted in

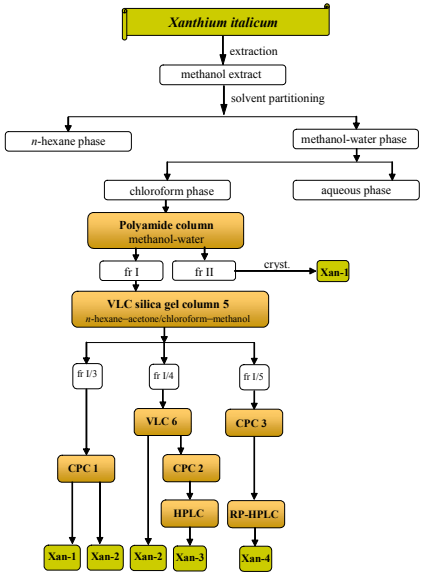


Figure 3. Isolation of xanthanolides from *X. italicum*

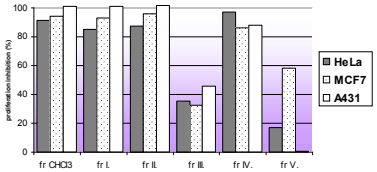


Figure 4. Antiproliferative activities of the $CHCl_3$ subfractions of leaves of *X. italicum* on the HeLa, MCF7 and A431 cell lines

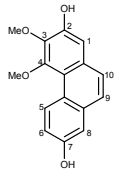
10 subfractions I/1-10, which were again tested on the HeLa, MCF-7 and A431 cell lines: subfractions I/2, I/3, I/4 and I/5 demonstrated significant activity.

In the following steps, even more selective methods (gel chromatography, CPC and preparative TLC, and HPLC) were applied for the purification of the selected active subfractions. Successful separations were achieved when various stationary and mobile phases were used in the subsequent steps of purification. Preparative TLC and gel chromatographic methods were applied for purification of the subfractions containing the few main components. For the purification of small subfractions, NP- and RP-HPLC were used. This on-line preparation technique resulted in the isolation of numerous constituents under mild conditions.

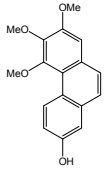
As a result of the isolation procedures, 14 compounds were isolated from the multicomponent samples: 10 phenanthrene-type compounds from *T. communis* (TC-2–TC-11) (**1-10**) together with *β*-sitosterine (TC-1), and 4 containing a xanthanolide skeleton from *X. italicum* (Xan-1–Xan-4) (**11-14**).

Characterization and structure determination of the isolated compounds

The isolated phenanthrenes were amorphous solids or crystals, while the xanthanolides were crystals, oils or gums. The structures of the isolated compounds were elucidated by means of spectroscopic methods. The UV spectra of the compounds from *T. communis* revealed the presence of the phenanthrene nucleus. Through the utilization of HREIMS spectrometry, the molecular compositions were determined. The most useful data concerning the chemical structures were furnished by the 1D and 2D NMR spectroscopy, including ^{1}H-^{1}H COSY, NOESY, HSQC and HMBC measurements. As a result of the detailed NMR studies, complete ^{1}H and ^{13}C chemical-shift assignments of compounds TC-5 (**4**), TC-7 (**6**), TC-10 (**9**), TC-11 (**10**) and Xan 1–4 (**11-14**) proved possible. The positions of the substituents in the phenanthrenes, and the relative configurations of the chiral centres in the xanthanolides were identified on the basis of NOESY experiments.



1 (TC-2)



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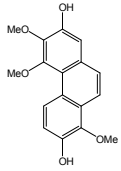
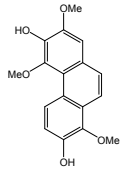
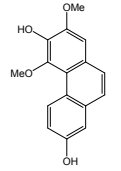


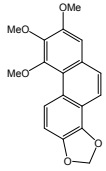
3 (TC-4)



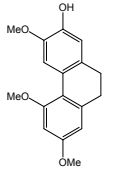
4 (TC-5)



5 (TC-6)



6 (TC-7)



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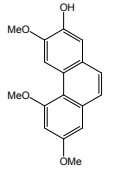
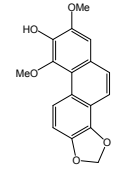


8 (TC-9)



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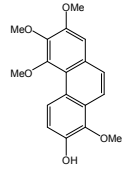
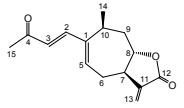
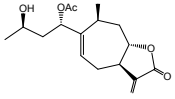


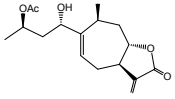
10 (TC-11)



11 (Xan-1)



12 (Xan-2)



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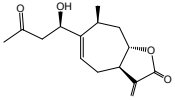

14 (Xan-4)

From the lipophilic phase of the rhizome of *T. communis* 9 phenanthrenes (**1-6, 8-10**) and one 9,10-dihydrophenanthrene (**7**) were identified. All the isolated phenanthrenes have a monomeric structure, and are methoxy- and (mainly) hydroxy-substituted. 2,3,4-Trimethoxy-7,8-methylenedioxyphenanthrene (**6**) and 3-hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (**9**) contain a methylenedioxy group at C(7)–C(8). 7-Hydroxy-2,3,4-trimethoxyphenanthrene (**2**) and 7-hydroxy-2,3,4,8-tetramethoxyphenanthrene (**10**) are new natural products, and a further 5 compounds [2,7-dihydroxy-3,4-dimethoxyphenanthrene (**1**), 2,7-dihydroxy-3,4,8-trimethoxyphenanthrene (**3**), 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**4**), 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**5**) and 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (**7**)] have now been described for the first time from *T. communis*.

Xanthatin, 4-epixanthanol, 4-epi-isoxanthanol and 2-hydroxyxanthinosin (**11-14**) isolated from *X. italicum* are members of the group of sesquiterpene lactones. This was the first identification of one of them, 2-hydroxyxanthinosine (**14**), from the plant.

Biological activities of the isolated compounds

The isolated phenanthrenes were tested for their cytotoxic activity on HeLa cells. Confusarin (**3**) exhibited the most potent cytotoxic activity (IC_{50} = 0.97 ± 0.009 μM), followed by 3-hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (**9**) (IC_{50} = 3.64 ± 0.12 μM) and 3,7-dihydroxy-2,4-dimethoxyphenanthrene (**5**) (IC_{50} = 6.66 ± 0.25 μM). 2,7-Dihydroxy-3,4-dimethoxyphenanthrene (**1**), 7-hydroxy-2,3,4-trimethoxy-phenanthrene (**2**), 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (**7**), 2-hydroxy-3,5,7-trimethoxyphenanthrene (**8**) and 7-hydroxy-2,3,4,8-tetramethoxyphenanthrene (**10**) displayed moderate cell growth-inhibitory effects (IC_{50} = 8.52–20.18 μM), whereas 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (**4**) and 2,3,4-trimethoxy-7,8-methylenedioxyphenanthrene (**6**) were found to be inactive. Although the low numbers of there compounds do not allow a thorough evaluation of structure-activity relationships, some conclusions can be drawn concerning the importance of various structural elements, as concerns tumor cell proliferation -

inhibitory activity. The cytotoxic potencies indicate that the C(9)–C(10) double bond is not essential for the cytotoxic effect, as the compounds 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (**7**) (IC_{50} = 14.21 ± 1.64 μM) and 2-hydroxy-3,5,7-trimethoxyphenanthrene (**8**) (IC_{50} = 11.49 ± 0.68 μM), which differing only in this structural feature, are almost equally effective. The number and positions of the methoxy groups on the phenanthrene skeleton seem to be crucial factors as regards the efficacy, similarly as for the conformationally less restricted analogues, the *cis*-stilbene combretastatins. Although the highly active combretastatins require a trimethoxy-substituted benzene ring in the molecule, our results reveal that this is not essential for the phenanthrenes, since 2,3,4-trimethoxy-7,8-methylenedioxyphenanthrene (**6**), containing 3 methoxy substituents, proved to be the least active (IC_{50} = >30 μM), and 2,7-dihydroxy-3,4,8-trimethoxyphenanthrene (**3**) (IC_{50} = 0.97 ± 0.009 μM) and 3-hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (**9**) (IC_{50} = 3.64 ± 0.12 μM), with only 2 methoxy groups, were the most effective in the MTT assay. Comparison of the effects of compounds **3, 6**, **9** and **10** suggests that the presence of a hydroxy group on either ring A or ring B is favourable relative to full alkoxy substitution. All of these findings demonstrate that the structure–activity relationships established for combretastatins cannot be directly applied to the congeners, *e.g.* to the conformational by constrained phenanthrenes.

The cytotoxic effects of the obtained xanthanolides were screened on three human cell lines (HeLa, A431 and MCF7), and it was found that xanthatin (**11**) demonstrated the highest activity against all of these cell lines (IC_{50} = 3.44–8.00 μM), and 2-hydroxyxanthinosine (**14**) displayed significant activity against HeLa cells (IC_{50} = 7.78 ± 1.21 μM). 4-Epixanthanol (**12**) and 4-epi-isoxanthanol (**13**) exerted moderate effects (IC_{50} 15.53−37.62 μM) against all three tumour cell lines. All these xanthanolides (**11-14**) contain an α-methylene-γ-lactone ring, which is generally regarded as a structural requirement of sesquiterpenes for cytotoxic activity. The most potent xanthatin (**11**) has an additional α,β-unsaturated carbonyl group in the side-chain; this structural feature presumably enhances the antitumor activity.

In conclusion, our results indicate that naturally occurring phenanthrenes, dihydrophenanthrenes and xanthanolides may be promising starting structures for antitumor drug development.

Acknowledgements

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I am especially grateful to my family for ensuring me a peaceful background.

List of publications

The thesis is based on the following publications:

1. Réthy B, **Kovács A**, Zupkó I, Forgo P, Vasas A, Falkay Gy, Hohmann J. Cytotoxic phenanthrenes from the rhizomes of *Tamus communis* *Planta Med.* **72**, 767-770 (2006)
   If: 1.848

2. **Kovács A**, Forgo P, Zupkó I, Réthy B, Falkay Gy, Szabó P, Hohmann J. Phenanthrenes and a dihydrophenanthrene from *Tamus communis* and their cytotoxic activity
   Phytochemistry **68**, 687-691 (2007)
   If: 2.322

3. **Kovács A**, Vasas A, Hohmann J.
   Natural phenanthrenes and their biological activity
   Phytochemistry **69**, 1084-1110 (2008)
   If: 2.322

4. **Kovács A**, Vasas A, Forgo P, Réthy B, Zupkó I, Hohmann J
   Xanthanolides with Antitumour Activity from *Xanthium italicum*
   Z. Naturforschung (in press)
   If: 0.756

Other publications:

1. Kovács A.
Citotoxikus fenantrének a *Tamus communis*-ból
Tudományos Diákköri Konferencia, SZTE GYTK – ÁOK
Szeged, 2005. February

2. Kovács A.
Citotoxikus fenantrének a *Tamus communis*-ból
XXVII. Országos Tudományos Diákköri Konferencia, Orvostudományi szekció, Gyógyszerésztudomány tagozat
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4. Kovács A.
Tumorsejtek szaporodását gátló fenantrének izolálása a *Tamus communis*-ból
VIII. Clauder Ottó Emlékverseny
Budapest, 2007. April 12-13.

5. Réthy B, **Kovács A**, Zupkó I, Hohmann J, Falkay Gy.
A *Tamus communis*-ból izolált fenantrének citotoxikus hatásának vizsgálata *in vitro*
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6. Vasas A, Rédei D, **Kovács A**, Forgó P, Zupkó I., Réthy B, Falkay Gy, Hohmann J.
Cytotoxic phenanthrenes from the rhizomes of *Tamus communis* L.
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Firenze, 2005. August 21-25.

7. **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
Dobogókő, 2005. October 13-15.

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

9. **Kovács A**, Vasas A, Réthy B, Zupkó I, Forgó P, Hohmann J.
Citotoxikus xanthanolidok izolálása a *Xanthium italicum* L.-ból
X. Tavaszi Szél Konferencia
Budapest, 2007. May 17-20.

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

11. **Kovács A**, Vasas A, Réthy B, Zupkó I, Forgó P, Hohmann J.
Antiproliferatív hatású szeszkviterpének a *Xanthium italicum*ból
Gyógynövény Szimpózium
Szeged, 2007. October 18-19.