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Activity-guided investigation of antiproliferative secondary metabolites of Asteraceae species

Ph.D. Thesis

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1. INTRODUCTION

In the past few decades, numerous useful antineoplastic drugs (e.g. taxoids, campthotecine, podophyllotoxin derivatives and *Vinca* alkaloids) have been discovered in higher plants by following up ethnomedicinal uses or the results of antitumour screening. Among currently available anticancer drugs, more than 60% of the new small molecular chemical entities are non-synthetic, a proportion which is much higher than in other areas of drug development. The ongoing search for naturally occurring anticancer agents is still very intense. The approval of ingenol-3-angelate, a cytotoxic diterpene ester from *Euphorbia peplus*, as a new drug (*Picato*®) indicated for the topical treatment of actinic keratosis, illustrates the success of this pursuit. Another perspective molecule, flavopiridol, a synthetic flavone derivative structurally based on rohitukine isolated from *Dysoxylum binectariferum*, is currently reported to be involved in 9 clinical trials ranging from phase I to phase II, covering leukaemias, lymphomas and solid tumours. Thapsigargin, a sesquiterpenoid from *Thapsia garganica*, has shown promise in a clinical trial as a chemotherapeutic drug against advanced solid tumours.

The process that leads from a plant to the production of a potential antitumour compound includes the primary screening of the plant extracts and the subsequent bioactivity-guided fractionation, comprising several consecutive steps of chromatographic separation, where each fraction obtained has to be submitted to bioassays in order to follow the activity. For the bioassays, a broad variety of cultured cancer cell lines are available as targets. After the isolation procedures, characterization and pharmacological evaluation of the pure compounds have to be carried out.

Plants can be selected for screening on the basis of ethnobotanical information or chemotaxonomic relationships to medicinal plants with anticancer properties. In the surveys by HARTWELL and GRAHAM on plants which had been reported to have ethnomedical uses for cancer-related diseases, data on about 300 species of Asteraceae were surveyed. The antitumour effects of Asteraceae species have been extensively studied, and sesquiterpene lactones or flavonoids have frequently been demonstrated to be responsible for their antitumour action. Several of these molecules are undergoing human studies as potential chemotherapeutic agents (artemisininoids, parthenolides and silibinin) or are regarded as good candidates for clinical trials (apigenin, eupatoriopcrin, helenanolides and xanthanolides).

Although appreciable experimental evidence and ethnobotanical data are available concerning the anticancer properties of Asteraceae species, only a few screening studies have been reported on the plants from this family, and none at all on the European species. The present work comprises an evaluation of the antitumour effects of plants from the Hungarian Asteraceae and detailed phytochemical investigations of *Conyza canadensis* (L.) CRONQ. and *Achillea millefolium* s.l.

2. AIMS OF THE STUDY

In recent years, the research group of the Department of Pharmacognosy at the University of Szeged has initiated a programme in collaboration with the Department of Pharmacodynamics and

Biopharmacy at the same university, with the purpose of obtaining potential antineoplastic compounds from the Hungarian flora. As part of this project, the aim of the present work was to carry out a comprehensive anticancer screening of Asteraceae species found in Hungary, and to identify the antitumour compounds present in certain selected plants. In order to achieve these goals, my main tasks were to

- review the literature on Asteraceae, concerning the chemistry and antitumour properties of the plants;
- collect plant material for the antitumour screening study of Asteraceae species native to Hungary;
- subject the collected plants to extraction with different solvents;
- examine the tumour cell proliferation-inhibitory activities of the extracts (carried out in the Department of Pharmacodynamics and Biopharmacy);
- select species with high antiproliferative activity, considered worthy of detailed phytochemical studies;
- collect plant material of the selected species for preparative work;
- extract the plant material and isolate the compounds responsible for the antiproliferative effects via bioactivity-directed fractionation, using various chromatographic techniques;
- elucidate the structures of the isolated compounds by NMR and MS methods, provide characteristic spectral data on the isolated new compounds, and supplement missing NMR data on the already-known constituents;
- evaluate the pharmacological potential of the isolated compounds (carried out in the Department of Pharmacodynamics and Biopharmacy).

3. MATERIALS AND METHODS

For the antiproliferative screening, Asteraceae species were collected between June and August 2004 from several regions of Hungary. For the phytochemical studies, the roots of *Conyza canadensis* (L.) were collected in the Southern Great Plain (Hungary) in September 2004. The plants were authenticated by TAMÁS RÉDEI (Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót). Herbs of *A. millefolium* s.l. were purchased in 2005 from Rózsahegyi Kft., Erdőkertes, Hungary. The air-dried raw materials were percolated with MeOH. The concentrated extracts were diluted with H_2O, and the solutions were extracted first with *n*-hexane and subsequently with $CHCl_3$ to furnish fractions of different polarity. For the antiproliferative screening, H_2O-soluble extracts were also prepared.

The isolation procedures were carried out by multistep chromatographic methods, including vacuum-liquid chromatography (VLC), rotation planar chromatography (RPC), preparative layer chromatography (PLC), gel filtration (GF) and high-performance liquid chromatography (HPLC).

Normal- or reversed-phased SiO_2, Al_2O_3 or Sephadex LH-20 were applied as stationary phases. The structure elucidation was carried out by means of spectroscopic (NMR, ESIMS and HREIMS) methods. In the course of the screening studies and the pharmacological assay of compounds from *A. millefolium*, antiproliferative effects were measured on 3 human cell lines [HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma) and A-431 (skin epidermoid carcinoma)] with the MTT assay. MRC-5 (non-cancerous human foetal lung fibroblast) cells were also applied to study the compounds of *C. canadensis*. Doxorubicin and cisplatin were used as positive controls.

4. RESULTS AND DISCUSSION

4.1. SCREENING OF THE HUNGARIAN ASTERACEAE FOR ANTITUMOUR EFFECTS

In the course of our preliminary screening for antiproliferative substances in the Asteraceae family, 50 species of the tribes Cynareae (13), Cichorieae (12), Astereae (6), Anthemideae (11), Inuleae (3) and Heliantheae (5) were evaluated *in vitro* against human tumour cell lines (HeLa, A-431 and MCF-7). A total of 420 extracts, obtained with *n*-hexane (A), $CHCl_3$ (B), aqueous MeOH (C) and H_2O (D) from different plant organs, were tested at a concentration of 10 μg/ml.

In summary, 41 extracts representing 21 species exerted ≥50% inhibition of the proliferation of at least one of the cell lines. For 22 species, a moderate (25–49.99%) cell growth inhibition was detected, while 7 plants were found to have no antitumour property. Extracts with ≥50% inhibitory potency were selected for additional measurements in order to determine IC_{50} values. In most cases, the selected extracts originated from the aerial plant parts and mainly fractions B were found to be active. The $CHCl_3$ extract from the roots of *C. jacea* was the most potent sample in the whole screen, with an IC_{50} value of 0.37 μg/ml on HeLa cells.

Relationship between bioactivity and traditional application

For *Conyza canadensis, Erigeron annuus*, *Ambrosia artemisiifolia, Helianthus annuus*, *Xanthium italicum, Arctium lappa, Cichorium intybus* and *Onopordum acanthium* the measured antiproliferative activities are in accordance with their traditional use as anticancer remedies. Other species, such as *Artemisia campestris*, *A. dracunculus*, *A. vulgaris, Tripleurospermum inodorum, Carduus acanthoides, Lactuca serriola*, *Sonchus oleraceus*, *Taraxacum officinale*, *Anthemis tinctoria, Matricaria chamomilla* and *Tragopogon pratensis* exerted only a marginal effect on the cell lines used or proved ineffective, in spite of their traditional use in cancer treatment.

Species worthy of activity-guided investigation

A survey on the literature data of the investigated species did not reveal any earlier pharmacological or phytochemical studies on secondary metabolites of *Anthemis ruthenica*, *Inula ensifolia*, *Centaurea biebersteinii*, *C. spinulosa* and *Cirsium vulgare*. With regard to their high tumour cell growth-inhibitory activities, these species can be regarded as promising sources of new cytostatic agents.

Certain plants found effective in our study have been more or less documented chemically or pharmacologically, but the active constituents have not been identified (*Achillea collina*, *Conyza*

canadensis, *Erigeron annuus*, *Centaurea jacea*, *Xanthium italicum* and *Lactuca viminea*), or presumably not completely exploited (*Artemisia asiatica, A. japonica*, *Onopordum acanthium* and *Ambrosia artemisiifolia*). These species are worthy of bioassay-guided investigation in order to isolate further active compounds responsible for antitumour activity.

4.2. Selection of plants for bioactivity-guided investigations

On the basis of the results of the preliminary screen and the literature survey of the tested species, *Conyza canadensis* and *Achillea collina* were chosen for more detailed phytochemical studies, with the aim of identification of their antitumour constituents. The screening results of *C. canadensis* demonstrated that the *n*-hexane-soluble fraction of the roots displayed high activities (62–71%), and the $CHCl_3$-soluble fraction of the roots induced a moderate inhibition of proliferation (39–48%) of all the tested cell lines. The anticancer effect of horseweed has not been evaluated previously. Earlier phytochemical studies focused primarily on the aerial parts, and only a few compounds had been described in the roots. In view of these results, investigation of both the n-hexane and $CHCl_3$ extracts of horseweed roots seemed to be promising.

The $CHCl_3$ fraction of the herb of *Achillea collina*, a member of the *A. millefolium* group, proved to be prominently active on HeLa (88.9%) and MCF-7 (53.9%). Yarrow is a known medicinal plant with well-documented chemistry. In folk medicine, it has been widely applied for the treatment of cancer-related diseases; however, experimental evidence of the antitumour properties of this plant is limited. For the preparative work, *Achilleae herba* of commercial origin (*Achillea millefolium* s.l.) was used as raw material.

4.3. Activity-guided investigations

The initial step of the processing of the plant materials included percolation with MeOH and subsequent liquid–liquid partitioning, yielding *n*-hexane and $CHCl_3$ fractions, which were subjected to a multistep chromatographic procedure under the guidance of MTT assays.

***Isolation of compounds from* Conyza canadensis**

Both the *n*-hexane and the $CHCl_3$ fractions of horseweed root were analysed (**Figure 1**). The crude separation of the *n*-hexane phase afforded 12 main fractions, among which 5 (A/IV–VIII) proved to be effective in the antiproliferative test and were therefore analysed in detail. RPC proved to be the most suitable method for subsequent procedures due to its high selectivity, speed and capacity. For final purification, PLC was also applied in 1 case when the separation with RPC was insufficient. From the active fractions, 7 substances (**EC-1**, **EC-4**–**7**, **EC-9** and **EC-10**) were isolated. In addition, **EC-3** was crystallized from the inactive fraction A/III. The VLC separation of the $CHCl_3$-soluble fraction resulted in 5 main fractions, 4 of which were found to be effective. Since the highly active fraction B/I contained earlier-isolated acetylenes (**EC-9** and **EC-10**), only the moderately active fractions B/II, B/III and B/IV were processed. Similarly as in the previous experiment, RPC was the most frequently used method. When a more selective method and mild conditions were necessary, RP-HPLC was applied. Chromatographic purification afforded **EC-14**–**16** and **EC-19.**

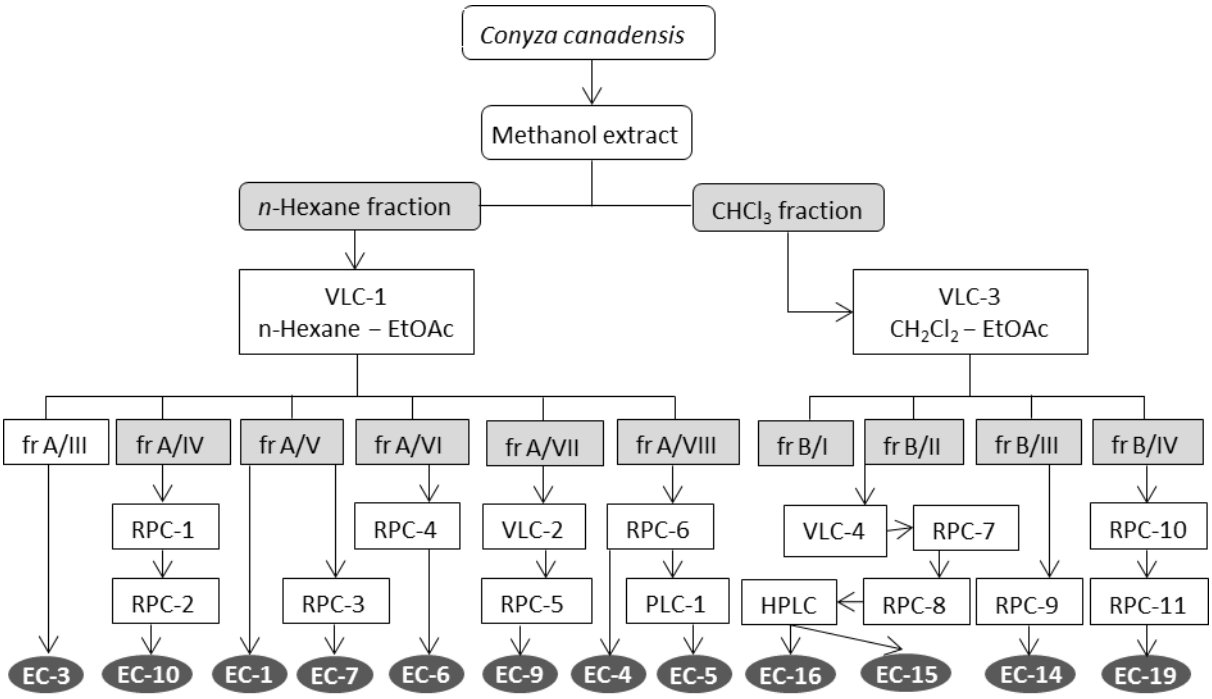


Figure 1. Isolation of compounds from *C. canadensis*

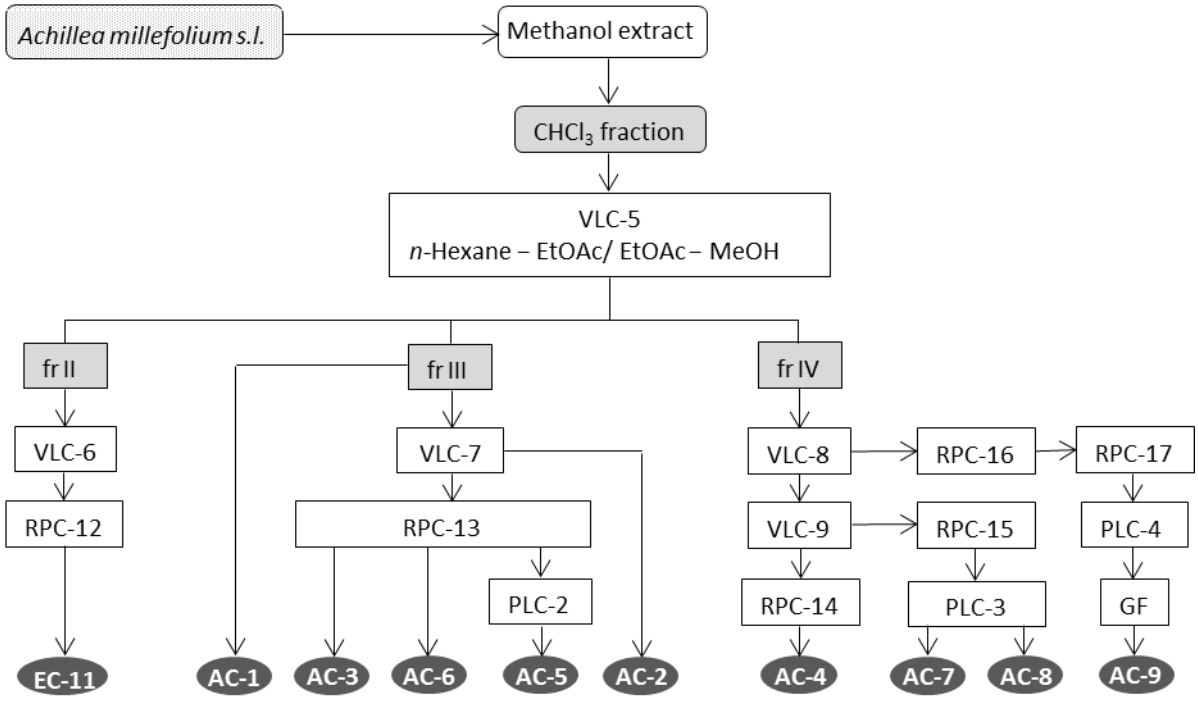


Figure 2. Isolation of compounds from *A. millefolium* s.l.

Isolation of compounds from* Achillea millefolium *s.l.

The crude fractionation of the $CHCl_3$-soluble fraction of yarrow furnished 8 main fractions (**Figure 2**). The active compounds were accumulated in 3 very complex fractions (II, III and IV) containing numerous substances of different chemical types. Accordingly, more selective methods were

required for further chromatography. The multistep application of VLC, RPC, PLC and GF on SiO_2 or Sephadex LH-20, with a variety of solvent systems permitted the separation of the diverse constituents and the removal of the redundant materials. Finally, the crystallization of the compounds facilitated the purification, allowing the isolation of 5 flavonoids (**AC-1**–**3**, **AC-5** and **AC-9**) and 5 sesquiterpene lactones (**AC-4**, **AC-6**–**8** and **AC-11**).

4.4. CHARACTERIZATION AND STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

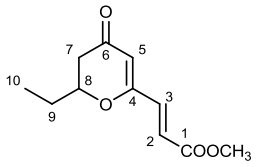
The most important data for the structure elucidation were gained from NMR measurements. 1D NMR (^{1}H NMR and JMOD) spectra were recorded for all substances; the already known compounds were identified by comparison of their NMR data with the literature values. 2D spectra (^{1}H,^{1}H-COSY, HSQC, HMBC and NOESY) and mass spectrometry were required for the analysis of the new structures, and in some cases for the already known compounds as well.

***Compounds in* Conyza canadensis**

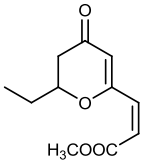
Twelve compounds were isolated from the roots of horseweed, among which 2 pyranone derivatives, conyzapyranone A (**EC-16**) and conyzapyranone B (**EC-15**), were described as new naturally occurring compounds. **EC-9** was identified as 4*E*,8*Z*-matricaria-*γ*-lactone and **EC-10** as 4*Z*,8*Z*-matricaria-*γ*-lacton, typical C_{10} acetylenes of the genus *Conyza*. The previously published NMR chemical shifts recorded in CCl_4 for these substances were supplemented with complete ^{1}H and ^{13}C NMR shift assignments in $CDCl_3$. For the fatty acid 9,12,13-trihydroxy-10*E*-octadecenoic acid (**EC-19**), described for the first time in this plant, we determined complete ^{13}C NMR data. Among triterpenes, the taraxerane-type taraxerol (**EC-6**) and the adianane-type simiarenol (**EC-7**) were described for the first time in *C. canadensis*, while the friedelane-type friedeline (**EC-3**) and epifriedelanol (**EC-1**) had already been isolated from this species. Stigmasterol and *β*-sitosterol, isolated as co-crystals (**EC-4**), spinasterol (**EC-5**) and the flavone apigenin (**EC-14**) are common plant constituents.

Compounds in* Achillea millefolium *s.l.

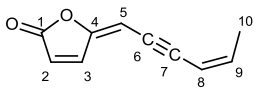
The structure analysis led to the identification of 5 flavonoids and 5 sesquiterpene lactones. The known artemetin (**AC-3**), casticin (**AC-5**), centaureidin (**AC-2**), apigenin (**AC-1**), luteolin (**AC-9**) and desacetylmatricarin (**AC-4**) were identified on the basis their spectral data. The *seco*-pseudoguaianolides **AC-7**, **AC-8** and **AC-11** were described for the first time in the genus *Achillea*. Complete ^{1}H NMR shift assignments were achieved for the stereoisomers paulitin (**AC-7**) and isopaulitin (**AC-8**), and the previously reported ^{13}C NMR shift assignments were corrected. For psilostachyin C (**AC-11**), the earlier published ^{13}C NMR data were supplemented. Sintenin (**AC-6**) was also isolated for the first time from the *A. millefolium* group. At the beginning of the structure analysis, the 1D NMR data on **AC-6** suggested its identity to millefin, a germacranolide with an *α*-acetyl function at C-8, as reported for *A. millefolium* by KASIMOV *et al*. in 1972. In order to determine the complete NMR assignment, 2D NMR measurements were performed, which clearly indicated that **AC-6** bears a *β*-acetyl substitutent, and led to the conclusion that the isolated compound is identical with sintenin, a germacranolide isolated previously from *Achillea micrantha*.



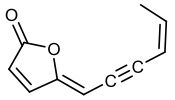
EC-16
conyzapyranone A



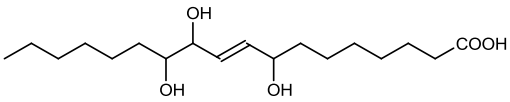
EC-15
conyzapyranone B



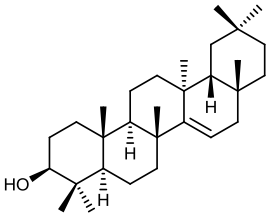
EC-9 4*E*,8*Z*-
matricaria-*γ*-lactone



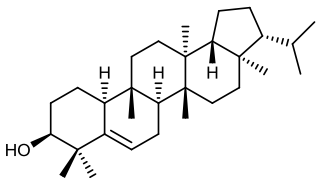
EC-10 4*Z*,8*Z*-
matricaria-*γ*-lactone



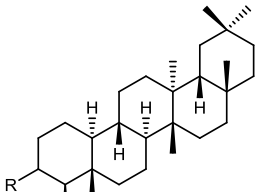
EC-19
9,12,13-trihydroxy-10*E*-octadecenoic acid



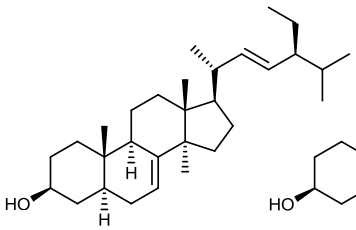
EC-6 taraxerol



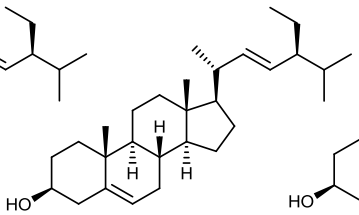
EC-7 simiarenol



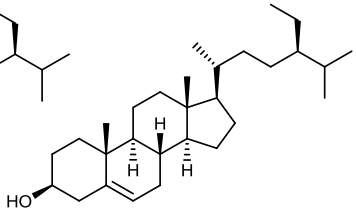
R
=O **EC-3** friedeline
β-OH **EC-1** epifriedelanol



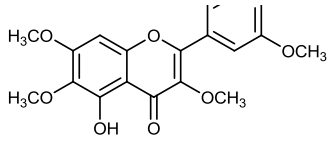
EC-5 spinasterol



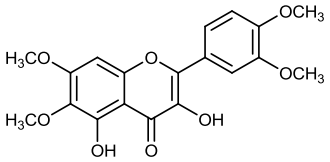
EC-4 stigmasterol



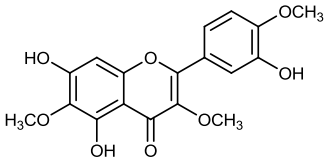
EC-4 *β*-sitosterol



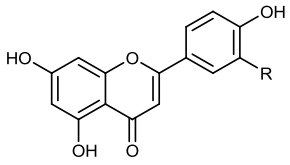
AC-3 artemetin



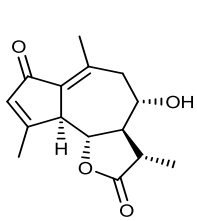
AC-5 casticin



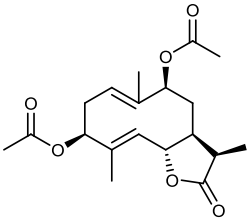
AC-2 centaureidin



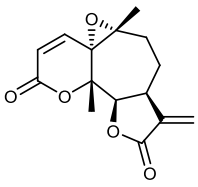
R
H **EC-14=AC-1** apigenin
OH **AC-9** luteolin



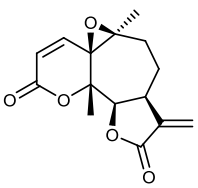
AC-4 desacetylmatricarin



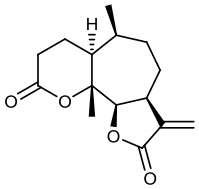
AC-6 sintenin



AC-7 paulitin



AC-8 isopaulitin



AC-11 psilostachyin C

4.5. PHARMACOLOGICAL ASSESSMENT OF THE ISOLATED COMPOUNDS

***Compounds in* Conyza canadensis**

Compounds from *C. canadensis* were tested on 3 cancerous (HeLa, MCF-7 and A-431) and 1 non-cancerous (MRC-5) cell line (**Table 1**) and it was found that the active compounds in horseweed

belong to different chemical classes (C_{10} acetylene, pyranone, triterpene, sterol and flavone-type compounds). Taraxerol, epifriedelanol, 4*Z*,8*Z*-matricaria-*γ*-lactone and apigenin exhibited the highest effects, to an extent comparable to that of the positive control cisplatin. The $CHCl_3$ fraction displayed only moderate antiproliferative activity in the preliminary assay; however, active substances (conyzapyranone A and B and apigenin) were isolated from it. The selective cytotoxic activity of new anticancer drug candidates, natural or synthetic, is one of their most critical pharmacological features. In our study, the IC_{50} values of taraxerol, epifriedelanol, spinasterol and apigenin indicate more pronounced toxicity on the investigated cancer cells than on MRC-5.

Compounds in* Achillea millefolium *s.l.

The pharmacological assessment of compounds of *A. millefolium* s.l. on 3 tumour cell lines (**Table 1**) revealed that flavonoids and sesquiterpenes can be involved in the antiproliferative action of the plant. The most active compound is centaureidin. Artemetin, a close analogue of centaureidin, is inactive, and casticin, containing 3-hydroxy and 3'-methoxy groups, is 1 order of magnitude less active than centaureidin. This finding is in accordance with the observation that hydroxy substituents on C-3' and C-5, and methoxy groups on C-3 and C-4' are necessary for maximum cytotoxic potency. Among the sesquiterpenoids, paulitin and isopaulitin exerted significant antiproliferative effects. Both compounds contain 2 *α,β*-unsaturated ($C–O–CH=CH_2$) systems, which was earlier found to determine the cytotoxicity of sesquiterpene lactones. Psilostachyin C, possessing only 1 $C–O–CH=CH_2$ moiety in the molecule, proved to be inactive. However, the presence of an epoxy functionality and its stereochemistry most probably play important roles in the antiproliferative potency, because of the significant difference in the activities of paulitin and its stereoisomer isopaulitin.

Table 1. Antiproliferative effects of the isolated compounds on tumour and non-tumour cell lines

Compound		IC_{50} values (μM)			
		HeLa	**MCF-7**	**A-431**	**MRC-5**
EC-9	4*E*,8*Z*-Matricaria-*γ*-lactone	24.46	18.74	22.81	73.75
EC-10	4*Z*,8*Z*-Matricaria-*γ*-lactone	27.03	6.90	32.45	28.10
EC-16	Conyzapyranone A	61.40	48.20	35.32	61.12
EC-15	Conyzapyranone B	31.83	46.00	37.13	79.63
EC-19	9,12,13-Trihydroxy-10*E*-octadecenoic acid	inactive	inactive	inactive	not tested
EC-3	Friedeline	inactive	inactive	inactive	not tested
EC-1	Epifriedelanol	16.39	61.43	5.40	inactive
EC-6	Taraxerol	inactive	inactive	2.65	inactive
EC-7	Simiarenol	inactive	inactive	inactive	not tested
EC-5	Spinasterol	13.93	26.50	13.66	71.14
EC-14	Apigenin	10.64	13.88	12.34	> 100.00
EC-4	Stigmasterol+ β-sitosterol	inactive	inactive	2.62*	11.31*

Table 1. *(continued)*		**HeLa**	**MCF-7**	**A-431**	**MRC-5**
AC-7	Paulitin	4.76	1.96	1.48	not tested
AC-8	Isopaulitin	11.82	13.68	6.95	not tested
AC-11	Psilostachyin C	inactive	inactive	inactive	not tested
AC-4	Desacetylmatricarin	inactive	inactive	inactive	not tested
AC-6	Sintenin	inactive	inactive	inactive	not tested
AC-3	Artemetin	inactive	inactive	inactive	not tested
AC-5	Casticin	1.29	1.52	3.58	not tested
AC-2	Centaureidin	0.08	0.13	0.35	not tested
AC-1	Apigenin	10.64	13.88	12.34	not tested
AC-9	Luteolin	7.59	32.88	26.26	not tested
Doxorubicin		0.15	0.28	0.15 (0.09*)	0.50 (0.29*)
Cisplatin		12.43	9.63	2.84 (0.85*)	4.11 (1.23*)

* In µg/ml

4.6 CHEMOTAXONOMIC AND BIOGENETIC ASPECTS

Conyzapyranone A and conyzapyranone B were identified as new natural compounds of horseweed. The structure of conyzapyranones, based on a C_{10} unsaturated carbon skeleton and having a carboxymethyl functionality, suggests a close relationship to C_{10} acetylenes, typical constituents of the genus *Conyza*. Incorporation studies have revealed that these compounds are biosynthesized from C_{18} acetylenes by multistep *β*-oxidation or by direct oxidation. It has been supposed that C_{10} lactones can originate from C_{10} acetylene acids, and other *O*-heterocyclic compounds may also be biosynthesized in a similar way. In the cases of conyzapyranone A and B, cyclization of the lachnophyllum methyl ester [$CH_3CH_2CH_2(C{\equiv}C)_2{-}CH{=}CH{-}COOCH_3$] precursor can be presumed; in this cyclization, the C-4–C-8 moiety of the molecule may be involved.

In contrast with our expectation that flavonols and pseudoguaianolides are the antitumour constituents of *A. millefolium*, a previous study on the benzene extract of this plant, collected in Japan, resulted in the isolation of 3 antiproliferative 1,10-*seco*-guaianolides, methyl achimillate A, B and C. These results suggested the great chemical variability of the *A. millefolium* aggregate, besides its morphological diversity.

Paulitin, isopaulitin and psilostachyin C were isolated for the first time from the *Achillea* genus. These *seco*-pseudoguaianolide-type sesquiterpene lactones were described earlier only from different *Ambrosia* species. Since the compounds were isolated from a commercial sample in our experiment, this raises the question of whether these substances are secondary metabolites of *Achillea millefolium* s.l. itself or arise from the impurity of the plant material. However, the yields of these compounds seem to disprove this possibility.

The study on the structure of the germacranolide sintenin furnished a surprising result. The structure of millefin, originally presumed for **AC-6**, was reported by KASIMOV *et al.* in 1972 for *A. millefolium*; the presence of this compound has never been confirmed in any plants by other authors. Although **AC-6** afforded ^{1}H and ^{13}C NMR data identical to those of millefin, our 2D NMR experiments proved the structure of sintenin for **AC-6**. The presence of millefin in yarrow is therefore doubtful.

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