JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 897–901

Received November 8, 2004
Accepted March 24, 2005
DOI 10.1211/0022357056398
ISSN 0022-3573

In-vitro antiproliferative effects on human tumour cell lines of extracts and jacaranone from *Senecio leucanthemifolius* Poiret

M. R. Loizzo, R. Tundis, G. A. Statti, F. Menichini and P. J. Houghton

Abstract

We have studied the cytotoxic activity of extracts and jacaranone from *Senecio leucanthemifolius* Poiret. Extracts from *S. leucanthemifolius* were able to inhibit the in-vitro proliferation of a series of human tumour cell lines. The dichloromethane extract demonstrated effective cytotoxic activity with an IC50 of 20.1 μg mL^{-1} on the large cell carcinoma cell line COR-L23. The ethyl acetate extract showed an IC50 value of 5.0 μg mL^{-1} and the butanol extract an IC50 value of 6.4 μg mL^{-1} on the same cell line. A major active constituent of the dichloromethane extract was shown to be jacaranone, which was demonstrated to have a very strong activity against all of the tumour cell lines with IC50 values between 2.86 and 3.85 μg mL^{-1}, although it did not account for all the activity observed. Constituents of *S. leucanthemifolius* extracts were identified by GC/MS analysis and NMR.

Department of Pharmaceutical Sciences, University of Calabria, I-87036 Arcavata di Rende, CS, Italy

M. R. Loizzo, R. Tundis, G. A. Statti, F. Menichini

Centre for Bioactivity Screening of Natural Products, Faculty of Pharmacy, King's College, FWB, 150 Stamford Street, London SE1 9NH, UK

P. J. Houghton

Correspondence: M. R. Loizzo, Department of Pharmaceutical Sciences, University of Calabria, I-87036 Rende (CS), Italy.
E-mail: mr.loizzo@unical.it

Funding and acknowledgements: The authors are grateful to Professor G. Cesca of the Botany Department at the University of Calabria, Italy, for identification of the plant material, and to Dr Alessandra Braca of the Department of Bioorganic Chemistry and Biopharmaceutics at University of Pisa for helpful NMR discussion. We thank Dr Tony Theobald of the Department of Pharmacy, King's College London for statistical advice. We are grateful for ERASMUS funding which enabled this work to be carried out.

Introduction

It is well established that natural products are an excellent source of chemical structures with a wide variety of biological activity, including anticancer properties (Cragg et al 1999). This has opened up new fields of investigation of potential antitumour compounds, some of which are already widely used in cancer chemotherapy (Arcamone et al 1980). Cytotoxic screening models provide important preliminary data to select plant extracts with potential antineoplastic properties. As part of a screening programme searching for natural products with anticancer properties from *Senecio* genus and other species, we have reported the cytotoxic activity of *Senecio leucanthemifolius* Poiret.

Senecio is the largest complex genus in the family Asteraceae and more than 1500 species have been reported with a world-wide distribution (Nordestam 1977). The pyrrolizidine alkaloids and furoeremophilanes are the most characteristic secondary metabolites of this genus, but sesquiterpenes have been found, while chalcones and flavonoids have been reported occasionally (Urones et al 1988). *Senecio* species have been used in folk medicine in the treatment of wounds and in anti-emetic, anti-inflammatory and vasodilator preparations (Bautista Peres et al 1991). Moreover some studies reported the cytotoxic activity of some species of *Senecio* (Barbetti et al 1986; Christov et al 2002).

Materials and Methods

Plant materials

The aerial parts of *Senecio leucanthemifolius* Poiret were collected in the flowering season in Calabria (Italy) in July 2002. The voucher specimen was identified by G. Cesca (Department of Botany, University of Calabria, Italy) and deposited at the Herbarium of University of Calabria (CLU) under the reference n. 4525. Dried and powdered aerial parts (974 g) of *S. leucanthemifolius* were extracted with

MeOH (3×5 L) at room temperature (146.96 g). The methanolic extract was further fractionated with *n*-hexane (10.53 g), dichloromethane (3.89 g), ethyl acetate (9.06 g) and butanol (7.35 g).

Culture conditions and sulforhodamine B assay

The protein-staining sulforhodamine B (SRB) assay was used for measurement of cell proliferation. The test is based on the estimation of cell number indirectly by providing a sensitive index of total cellular protein content which is linear to cell density (Skehan et al 1990; Rubinstein et al 1990). SRB is an anionic protein stain containing two sulphonic groups that bind electrostatically to basic amino acid residues of cellular protein under mildly acid conditions. The bound dye can be quantitatively extracted from cells by weak bases and solubilized for spectrophotometry. Four cancer cell lines (large cell carcinoma COR-L23 (ECACC No.: 92031919), colorectal adenocarcinoma Caco-2 (ATCC No.: HTB-37), amelanotic melanoma C32 (ATCC No.: CRL-1585), and hepatocellular carcinoma HepG-2 (ECACC No.: 85011430)) and one normal cell line (human fetal lung MRC-5 (ATCC No.: CCL-171)) were used in this experiment. The COR-L23 and C32 cells were cultured in RPMI 1640 medium, while the MRC-5, Caco-2, and HepG-2 cells were cultured in D-MEM medium. Both media were supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin. The cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. The optimal plating density of each cell line was determined over a concentration range of $2 \times 10^4 - 5 \times 10^4$ to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 540 nm and cell number where analysed by the SRB assay. For the assay, cells were detached with 0.1% trypsin–EDTA to make a single-cell suspension, and viable cells were counted by trypan blue exclusion in a haemocytometer and diluted with medium to give a final concentration of $2–5 \times 10^4$ cell/well.

This cell suspension (100 μL/well) was seeded in 96-well microtitre plates and incubated to allow for cell attachment. After 24 h the cells were treated with the serial dilutions of the extracts. Each extract was initially dissolved in an amount of dimethyl sulfoxide (DMSO) and further diluted in medium to produce six concentrations. Each concentration (100 μL/well) was added to the plates in six replicates to obtain final concentrations of 5, 10, 20, 30, 50, 100 $\mu g\,mL^{-1}$ for extracts and 0.25, 0.5, 1, 2.5, 5, 12.5, 25, 50 $\mu g\,mL^{-1}$ for jacaranone. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of the solvent (DMSO), the same as in the solvent-control wells. The final volume in each well was 200 μL. The plates were incubated for the selected exposure time of 72 h. At the end of exposure time 100 μL ice-cold 40% trichloroacetic acid (TCA) was added to each well, left at 4°C for 1 h, and washed five times with distilled water. The TCA-fixed cells were stained for 30 min with 50 μL 0.4% (w/v) sulforhodamine B in 1% HOAc. The plates were washed five times with 1% HOAc and air dried overnight. Vinblastine sulfate salt was used as positive control. On the day of plate reading, bound dye was solubilized with 100 μL 10 mM Tris base (Tris[hydroxymethyl]aminomethane). All products were purchased from Sigma, UK. The absorbance of each well was read on a Spectra Max plate reader at 564 nm. Cell survival was measured as the percentage absorbance compared with the control (non-treated cells). Results for the experiments and compounds tested are given in Table 1.

Statistical analysis

All values were expressed as means ± standard deviation of the mean (s.d.). Differences within and between groups were evaluated by nonparametric analysis of variance tests (Friedman and Kruskal–Wallis) completed with a multi-comparison Dunn test using GraphPad software. A P value of < 0.05 was considered significant. The inhibitory concentration 50% (IC50) was calculated from a dose–response curve obtained by plotting the percentage of inhibition vs the concentrations with the use of GraphPad Prism 4 software.

GC/MS analysis

The identification of the compounds from extracts of *S. leucanthemifolius* was made by GC-MS analysis on a Hewlett-Packard 6890N gas chromatograph equipped with a methylsilicone SE-30 capillary column (30 m × 0.25 mm id × 0.25-μm film thickness) and interfaced with a Hewlett

Table 1 Cytotoxic activity of extracts and jacaranone from *Senecio leucanthemifolius* Poiret (IC50 $\mu g\,mL^{-1}$)

Cell type	MeOH	*n-Hexane*	CH_2Cl_2	EtOAc	BuOH	Jacaranone	Vinblastine
MRC-5	> 100*	> 100	> 100	> 100	> 100	> 50*	37 ± 0.44
Caco-2	> 100*	> 100	36.37 ± 0.78	> 100	69.46 ± 0.88	3.13 ± 0.97*	69 ± 0.56
COR-L23	> 100*	68.36 ± 0.39	20.07 ± 0.89	5.02 ± 0.34	25 ± 0.66	3.85 ± 0.64*	45.45 ± 0.73
C32	> 100*	43.91 ± 0.86	> 100	78.96 ± 0.34	> 100	2.06 ± 0.32*	3 ± 0.34
HepG2	> 100*	30.88 ± 0.54	85.73 ± 0.32	34.61 ± 0.72	6.36 ± 0.44	2.56 ± 0.76*	55.6 ± 0.83

Vinblastine was used as positive control. Data are given as the mean of at least three independent experiments ± s.d. Differences within and between groups were evaluated by nonparametric analysis of variance tests (Friedman and Kruskal–Wallis) completed with a multi-comparison Dunn's test. *$P < 0.01$.

Packard 5973N Mass Selective Detector, operating in Electron Ionization (E.I.) (70 eV). Carrier gas was helium. The 'solvent delay', the time gap of a given analysis in which the mass spectrometer is turned off, was 3 min. A 1-μL sample was dissolved in the appropriate solvent and injected into the gas chromatograph. The injector and detector temperatures for the gas chromatograph were 250 and 280°C, respectively. The analytical conditions worked with the following programme: oven temperature was programmed from 60 to 280°C at a rate of 16°C min^{-1}; the final temperature of 280°C was held for 10 min. Identification of the compounds from *S. leucanthemifolius* extracts were based on the comparison of the mass spectral data on computer matching against Wiley 138, Wiley 275 and NIST 98 and a home-made mass spectral library built from pure substances. The results from these analyses are summarized in Table 2, where indication of the sample from which each component has been identified is reported.

Table 2 Compounds present within *Senecio leucanthemifolius* extracts and identified by GC-MS

Extracts	Identified compounds
n-Hexane	Docosanoic acid, methyl ester
	Eicosanoic acid, methyl ester
	Octadecanoic acid, methyl ester
	Hexadecanoic acid
	Hexadecanoic acid, methyl ester
	Hexadecanoic acid, ethyl ester
	2-Hexadecen-1-ol,3,7,11,15-tetramethyl, [R-[R*,R*-(E)]
	9,12-Octadecadienoic acid (Z,Z)
	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)
	9,12,15-Octadecatrienoic acid, ethyl ester (Z,Z,Z)
	11,14-Octadecadienoic acid, methyl ester
	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester
	Ethyl linoleate
	a-Amyrin
	β-Amyrin
	Lup-20(29)-en-3-one
	Vitamin E
	Stigmasta-5,22-dien-3-ol (3β, 22E)
	(22R,24S)-22,24-dimethylcholesterol
	Ergost-5-en-3-ol, (3β, 24R)
Dichloromethane	Senecionine
	Integerrimine
	Jacaranone
	1,3-Benzenediol
	4,4,5,6-Tetrahydro-2,8(3H,1H)-naphthalenedione
	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl ester)
	Pentadecanoic acid, 14-methyl-methylester
Ethyl acetate	Propanedioic acid, dimethyl ester
	9,12,15-Octadecatrienoic acid, methyl ester
Butanol	Butanedioic acid, dimethyl ester
	Butanedioic acid, hydroxy-, didimethyl ester

Result and Discussion

Composition of extracts

To identify the inhibitory compounds present within the *S. leucanthemifolius* extracts silica gel TLC was carried out and specific spray reagents used for various compound types (Wagner & Bladt 1996). The *n*-hexane extract was developed with *n*-hexane:acetone (6:4) and then treated with vanillin-sulphuric acid reagent (VS) highlighting the presence of terpenoids. Alkaloids were detected in dichloromethane extract eluting with *n*-hexane:EtOAc (6:4), immediately followed by spraying with Dragendorff's reagent. The ethyl acetate and butanol extracts were developed with EtOAc:HCOOH:AcOH:H_2O (100:10:10:20) and EtOAc:*i*-Prop:NH_4OH (80:15:5), respectively, and than sprayed with Natural Products reagent-polyethylene glycol reagent revealing the presence of flavonoids when examined under UV light at 365 nm.

Gas-chromatography/mass-spectrometry (GC/MS) analysis of all extracts was performed. *S. leucanthemifolius* extracts were diluted to a final volume of 1 mL with methanol (approximately 1 mg mL^{-1}). A 1-μL sample of each solution was injected into the gas chromatograph with an appropriate microsyringe, chromatographed and analysed with a quadrupole mass spectrometric detector. A total of 31 compounds were identified (Table 2). These were known but had not been reported previously for *S. leucanthemifolius*.

The dichloromethane extract, successively, was subjected to repeated column chromatography over silica gel (Merck) 20–45 μm (CH_2Cl_2/MeOH 85:15). Column fractions were assayed and combined according to their TLC profile on Silica gel (Merck)/CH_2Cl_2:MeOH 9:1 and detected by Dragendorff's reagent and H_2SO_4 50% v/v, to afford senecionine ([Z] 7-ethylidene-10-hydroxy-9,10-dimethyl-2,3,4,4a,7,8,9,10,13,13b-decahydro-5,12-dioxa-2a-aza-cyclododeca [cd] pentalene-6,11-dione) (5 mg; 0.13% compared with the weight of extract), integerrimine ([E] 7-ethylidene-10-hydroxy-9,10-dimethyl-2,3,4,4a,7,8,9,10,13,13b-decahydro-5,12-dioxa-2a-aza-cyclododeca [cd] pentalene-6,11-dione) (3.6 mg; 0.09% compared with the weight of extract) and jacaranone (1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetic acid methyl ester) (69 mg; 1.77% compared with the weight of extract) (Figure 1). The structures of the compounds were determined on the basis of their spectral data (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) and were found to be identical with those described previously (Ogura et al 1976; Roeder & Bourauel 1993).

Cytotoxic activity

Approximately 60% of all drugs in clinical trials against cancer are either natural products or their modified versions often displaying cytotoxic effects (Cragg & Newman 2000). Cytotoxicity is commonly used as a target for discovery of novel anticancer compounds and the sulforhodamine B (SRB) assay, used in this study, is commonly employed. This assay is popular since it is relatively inexpensive, easy to perform and allows for a large number of samples to be

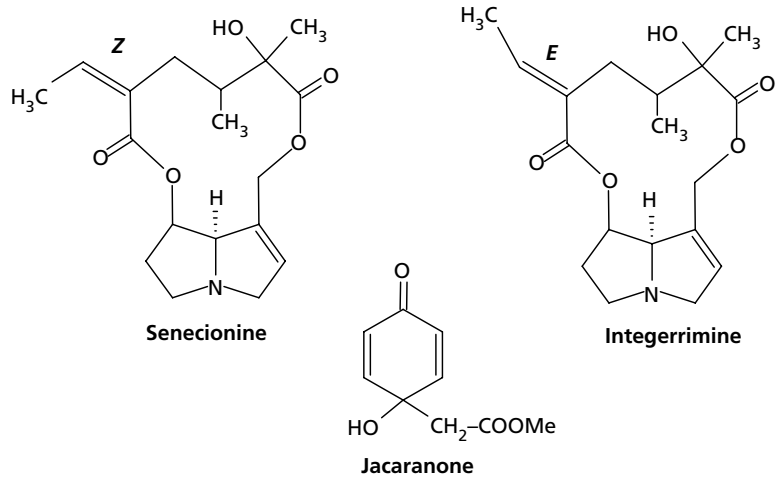

Figure 1 Compounds isolated from *Senecio leucanthemifolius* Poiret.

processed in a short time. The latter is of an important advantage as different cell lines have variable susceptibility to different extracts or compounds. The cytotoxic response will be dependent on the cell line tested and obtained results cannot be applied to other cell lines (Monks et al 1991). IC50 values of 4 and 20 $\mu g\,mL^{-1}$ for pure compounds and extracts, respectively, were set up as guidelines for classification of cytotoxic activity (Suffness & Pezzuto 1991).

Extracts from *S. leucanthemifolius* were able to inhibit the in-vitro proliferation of four human tumour cell lines (the large cell carcinoma COR-L23, colorectal adenocarcinoma Caco-2, amelanotic melanoma C32, and hepatocellular carcinoma HepG-2) and human fetal lung cell line MRC-5. All results are summarized in Table 1. Our data demonstrated that *S. leucanthemifolius* inhibited the viability of several tumour cell lines in a concentration-dependent manner.

The ability of the dichloromethane extract to inhibit proliferation of the large cell carcinoma (IC50 20.1 $\mu g\,mL^{-1}$) and colorectal adenocarcinoma (IC50 36.37 $\mu g\,mL^{-1}$) may be ascribed to one or more of the compounds identified in this extract, which include alkaloids and quinonoids. A potentially active compound is jacaranone which showed a strong inhibition on proliferation of tumour cell lines used in this study with IC50 values ranging from 2.06 (against C32 cells) to 3.85 $\mu g\,mL^{-1}$ (against COR-L23). This compound possesses a higher cytotoxicity than vinblastine which gave an IC50 value of 3.0 $\mu g\,mL^{-1}$ against C32, the most sensitive cell line.

Jacaranone was isolated for the first time from *Jacaranda caucana* (Ogura et al 1976), a member of the family Bignionaceae. This compound had shown activity against P-388 lymphocytic leukaemia (T/C 165 at 2 $mg\,kg^{-1}$) and Eagle's 9KB carcinoma cells, giving an IC50 value of 2.1 $\mu g\,mL^{-1}$ (Ogura et al 1976, 1977).

Jacaranone is active against promastigotes of *L. amazonensis* at an ED50 of 0.02 mM (Chan-Bacab & Peña-Rodríguez 2001), has antibacterial activity (Cabezas et al 1991) and inhibits human immunodeficiency virus reverse transcriptase (HIV) (Tan Ghee et al 1991).

Although jacaranone clearly showed activity, the activity of the CH_2Cl_2 extract could not be explained only in terms of its jacaranone content. Table 3 compares the IC50 value of the CH_2Cl_2 extract and its jacaranone content. The concentration of jacaranone in all cases was below its IC50 value. Other active substances might have been present or other compounds present might have had a synergistic effect on the activity of jacaranone, but this requires further study.

The *n*-hexane extract exhibited interesting activity against hepatocellular carcinoma (IC50 value 30.88 $\mu g\,mL^{-1}$). The ethyl acetate and butanol extracts which were shown to contain polyphenolic compounds in TLC analysis exhibited a strong antitumoral activity against COR-L23 (IC50 value 20.07 $\mu g\,mL^{-1}$) and HepG2 (IC50 value 6.36 $\mu g\,mL^{-1}$). Considering the IC50 values reported in Table 1, we found significant differences using Friedman ($F = 16.57$, $^*P < 0.01$) and Kruskal–Wallis ($K = 15.35$, $^*P < 0.01$) tests, but not when the post hoc Dunn's test ($P > 0.05$) was used, except when the activity of the methanol extract and the activity of jacaranone were compared ($P < 0.01$).

The inhibitory activity of *S. leucanthemifolius* extracts and of jacaranone on the proliferation of cancer cell lines but not on the normal human fetal lung MRC-5 suggested a specific

Table 3 Comparison of the cytotoxicity activity of the CH_2Cl_2 extract and of its jacaranone content (jacaranone = 1.77% w/w)

Cell type	IC50 CH_2Cl_2	Corresponding concn jacaranone ($\mu g\,mL^{-1}$)	IC50 Jacaranone
MRC-5	> 100	> 1.77	> 50
Caco-2	36.37 ± 0.78	0.62	3.13 ± 0.97
COR-L23	20.07 ± 0.89	0.34	3.85 ± 0.64
C32	> 100	> 1.77	2.06 ± 0.32
HepG2	85.73 ± 0.32	1.54	2.56 ± 0.76

Data are given as the mean of at least three independent experiments ± s.d. IC50 values were expressed as $\mu g\,mL^{-1}$.

mechanism of action interfering with abnormal proliferation. These results were in accordance with the cytotoxic activity of other members of this family (Barbetti et al 1986).

Further work is necessary on the isolation and identification of active cytotoxic compounds from the ethyl acetate and butanol extracts of *S. leucanthemifolius*.

References

Arcamone, F., Cassinelli, G., Casazza, A. M. (1980) New antitumor drugs from plants. *J. Ethnopharmacol*. **2**:149–160

Barbetti, P., Fardella, G., Chiappini, I., Menghini, A., Furlani Candiani, A., Scarcia, V. (1986) In vitro cytostatic activity of chromatographic fractions of extracts from some species of *Compositae*. *Arzneimittelforschung* **36**: 425–427

Bautista Peres, J., Stubing, G., Figuerola, R. (1991) *Guia de las Plantas Medicinales de la Communidad Valenciana*. Las Provincial, Valencia.

Cabezas, J. A., Ciccio, J. F., Hidalgo, G., Echandi, G. (1991) Synthesis and antibacterial activity of jacaranone and ester side chain analogs. *Revista Latinoamericana de Quimica* **22**: 49–52

Chan-Bacab, M. J., Peña-Rodríguez, L. M. (2001) Plant natural products with leishmanicidal activity. *Nat. Prod. Rep*. **18**: 674–688

Christov, V., Mikhova, B., Alexandrova, R., Dimitrova, D., Nikolova, E., Evstatieva, L. (2002) Alkaloids from the roots of *Senecio macedonicus* Griseb. *Z Naturforsch [C]* **57**: 780–784

Cragg, G. M., Newman, D. J. (1999) Discovery and development of antineoplastic agents from natural sources. *Cancer Invest*. **17**: 153–163

Cragg, G. M., Newman, D. J. (2000) Antineoplastic agents from natural sources: achievements and future directions. *Expert Opin. Investig. Drugs* **9**: 2783–2797

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A. (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst*. **83**: 757–766

Nordestam, B. (1977) *The biology and chemistry of the compositae*. Academic Press Inc., London, pp 799–830

Ogura, M., Cordell, G. A., Farnsworth, N. R. (1976) Potential anticancer agents. III. Jacaranone, a novel phytoquinoid from *Jacaranda caucana*. *Lloydia* **39**: 255–257

Ogura, M., Cordell, G. A., Farnsworth, N. R. (1977) Potential anticancer agents. IV. Constituents of *Jacaranda caucana* Pittier (Bignoniaceae). *Lloydia* **40**: 157–168

Roeder, E., Bourauel, T. (1993) Pyrrolizidine alkaloids from *Senecio leucanthemifolius* and *Senecio rodriguezii*. *Natural Toxins* **1**: 241

Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., Boyd, M. R. (1990) Comparison of in vitro anticancer-drug-screening data generated with a tretrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl. Cancer Inst*. **82**: 1113–1118

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., Boyd, M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst*. **82**: 1107–1112

Suffness, M., Pezzuto, J. M. (1991) Assays related to cancer. Drug discovery. In: Hostettman, K. (ed.) *Methods in plant biochemistry: assays for bioactivity*. Vol. 6, Academic Press Ltd

Tan Ghee, T., Pezzuoto, J. M., Kinghorn, A. D., Hughes, S. H. (1991) Evaluation of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. *J. Nat. Prod*. **54**: 143–154

Urones, J. G., Barcala, P. B., Marcos, I. S., Moro, R. F., Esteban Lòpez, M., Rodriguez, F. (1988) Pyrrolizidine alkaloids from *Senecio gallicus* and *Senecio adonifolius*. *Phytochemistry* **27:** 1507–1510

Wagner, H., Bladt, S. (1996) *Plant drug analysis*. Springer, Germany