THE EFFECT OF CERTAIN PLANT EXTRACTS CONTAINING PYRROLIZIDINE ALKALOIDS ON *LACTUCA SATIVA* RADICLE GROWTH

OANA-CRISTINA ŞEREMET, O.T. OLARU**#, D. BĂLĂLĂU***, SIMONA NEGREŞ**

* Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
** Department of Botany and Cellular Biology, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania,
#e-mail: octavian.olaru@umf.ro
*** Department of Toxicology, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

Abstract: Pyrrolizidine alkaloids (PAs) are toxins synthesized by plants, known to be hepatotoxic, genotoxic and carcinogenic. *Tussilago farfara* (coltsfoot), *Petasites hybridus* (common butterbur), *Senecio vernalis* (eastern groundsel) and *Symphytum officinale* (comfrey) are species traditionally used in phytotherapy that besides the therapeutic compounds contain PAs. The total PAs and the corresponding N-oxides content of the solid plant extracts were measured using Ehrlich's method with senecionine as a standard substance. The highest content of PAs was found in *Senecio vernalis* extract (424.92±9.81 mg%), followed by *Symphytum officinale* extract (150.24±10.35 mg%) and *Petasites hybridus* extract (0.021±0.00091 mg%). The lowest concentration was found in *Tussilago farfara* extract (0.0097±0.00072 mg%). In order to assess the toxicity of the extracts on *Lactuca sativa*, inhibitory concentrations 50% (IC_{50}) of the root elongation were calculated. All extracts inhibited the elongation of the *Lactuca sativa* radicle**s**. *Senecio vernalis* extract exhibited the highest toxicity, whilst *Symphytum officinale* extract, having the second highest concentration of PAs, has the highest IC_{50}. Further studies should be performed in order to determine whether the inhibitory effect is due to the PAs content and how it is influenced by the other components of the extracts.

Key words: Pyrrolizidine alkaloids, phytotoxicity, Ehrlich's method, *Lactuca sativa*.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are toxins synthesized exclusively by plants. About 6000 plant species worldwide (3% of all flowering plants) may contain PAs. These alkaloids are commonly found in Boraginaceae, Asteraceae and Fabaceae

Received: January 2014;
February 2014.

ROMANIAN J. BIOPHYS., Vol. 24, No. 1, P. 1–9, BUCHAREST, 2014

families [19]. *Tussilago farfara* L. (coltsfoot)*, Petasites hybridus* L. (Gaertn., Mey. et Scherb.) (common butterbur), *Senecio vernalis* (Waldst. et Kit.) (eastern groundsel) – of *Asteraceae* family, and *Symphytum officinale* L. (comfrey) of *Boraginaceae* family, are species traditionally used in phytotherapy that besides the therapeutic compounds contain toxic PAs [17].

The toxic PAs are based on two-fused five membered heterocyclic rings (necine base), that possess a double bond in the 1, 2 position (Fig. 1). Usually, in plants, the PAs (as free bases) coexist with the corresponding N-oxides, in different proportions. In the gastrointestinal tract of animals the N-oxides are reduced to the free bases and have a similar toxicity [1, 17].

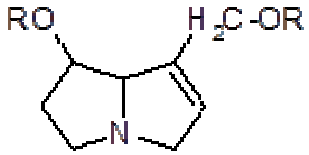

Fig. 1. General structure of a pyrrolizidine alkaloid.

Acute or chronic exposure to PAs can cause liver damage manifested mainly as acute venous-occlusive disease that may lead to cirrhosis. They also possess genotoxic (mutations, sister chromatid exchanges, chromosomal aberrations) and carcinogenic properties [11, 16].

Several methods are used for the spectrophotometric quantification of PAs. Among these methods, the one that uses the Ehrlich reagent has the advantage of being able to quantify very low levels of alkaloids (down to a few micrograms). This method is specific for the alkaloids that have a double bound in the 1 – 2 position of the necine base, alkaloids known to be hepatotoxic. Also, this method can make distinction between PAs and their N-oxides, which react directly with Ehrlich's reagent [4].

Phytobiological tests are often used in the screening of toxicity of plant extracts, natural and synthetic compounds [6, 14]. These tests can provide useful information about cytotoxicity and genotoxicity. The assay based on *Lactuca sativa* is used as a pre-screening in toxicology studies in higher plants, because of its advantages: quicker outcome, simplified operative procedure, good reproducibility and repeatability, low costs [3, 15].

The aim of the present study was to determine whether there is a connection between the total PAs content of the above mentioned species and their phytotoxicity.

MATERIALS AND METHODS

SPECTROPHOTOMETRIC ASSAY

Principle

In the presence of hydrogen peroxide, the PAs are converted to the corresponding N-oxides and acylated with acetic anhydride. By heating, acetic acid is removed and a pyrrole derivative is formed. This pyrrole reacts with 4-dimethylaminobenzaldehyde (Ehrlich's reagent) to form a violet-colored complex which can be spectrophotometrically measured. By omitting the first step (conversion of PAs to their N-oxides by hydrogen peroxide) only N-oxides will be determined [4, 5].

Materials

Standard solutions in methanol were prepared from a stock solution of senecionine (200 mg/mL, Carl Roth, Germany).

Plant Material. Coltsfoot leaves, common butterbur roots teas (produced by Stef Mar Ltd., Valcea) and comfrey roots tea (produced by Fares, Orastie) were purchased from retail stores. *S. vernalis* aerial part was harvested in May 2013, from Craiova Botanical Gardens (Dolj County), naturally dried and conserved in laboratory conditions. A voucher specimen is deposited at the Department of Botany, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania.

Plant Extracts. The extracts were obtained using a modified method described by R. Lebada *et al.* [2]. Briefly, the dried plants were pulverized and sieved (sieve No. V) and 20 g of each plant material were refluxed twice for 2 h with 1000 mL of 50% methanol acidified with citric acid to pH 2–3. The combined extracts have been evaporated, under reduced pressure with a Rotavapor system (Buchi, Switzerland) to about 300 mL and atomized with a Mini Spray Dryer B-290 (Buchi, Switzerland). The solid plant extracts were coded as: SYM (*Symphytum officinale*); SEN (*Senecio vernalis*); PET (*Petasites hybridus,*); TUSS (*Tussilago farfara*).

For the total PAs content assay, 2 g of SYM and SEN and 4 g of PET and TUSS were dissolved in 30 mL of methanol acidified with 50% citric acid to pH 2–3. After complete dissolution, the solution was purified twice by liquid-liquid extraction with 30 mL of chloroform and twice with 30 mL diethyl ether. The aqueous solutions were alkalinized with aqueous ammonia 25% (pH 9–10) and the PAs were extracted three times with 30 mL of chloroform. The chloroform solutions were reduced under pressure, the residue was dissolved in 2 mL methanol and filtered through a 0.2 μm syringe adaptable filter (Pall Life Science, USA).

Reagents. Oxidation reagent: 20 mL of methanol (Merck, Germany) were mixed with 0.20 mL of hydrogen peroxide 30% (Merck, Germany) containing sodium pyrophosphate (Merck, Germany) 5 mg/mL as a stabilizer, 0.20 mL of ethylene glycol (Merck, Germany) and 20 mg of butylated hydroxytoluene (Sigma-Aldrich, USA)

Diglyme: Diethylene glycol dimethyl ether (Merck, Germany) containing 5 mg/mL butylated hydroxytoluene (Sigma-Aldrich, USA)

Acetic anhydride (Sigma Aldrich, USA) was redistilled and the fraction boiling between 136–139 °C was collected

Modified Ehrlich's reagent: 4 mL of 14% boron trifluoride in methanol (Acros Organics, Belgium) is diluted with 36 mL of absolute ethanol (Scharlau Chemicals, Spain) and 0.8 g of 4-dimethylaminobenzaldehyde (Sigma Aldrich, USA) are added.

Method

For the preparation of the calibration curve, volumes of the working standard solutions, corresponding to 2.5–20 µg senecionine were evaporated under nitrogen jet with TechneDry-Block DB-3D (BibbyScientific Inc., Great Britain); 0.5 mL oxidizing reagent was added and the test tubes were left in the boiling water bath WNB 10 (Memmert, Germany) for 20–30 min. 1 mL diglyme and 0.1 mL acetic anhydride were added and the tubes were heated again in the water bath for 1 minute. After cooling the tubes at room temperature, 1 mL of modified Ehrlich reagent was added and the tubes were heated in a water bath at 55–60 °C for 4–5 minutes. The samples were transferred in volumetric flasks and acetone (Chimopar, Romania) was added up to 10 mL.

For assessing total alkaloid content**,** the samples were evaporated under nitrogen stream and the same procedure was followed as described in the preparation of the calibration curve. To determine N-oxides content**,** adding of oxidizing reagent was omitted.

All the measurements were performed on a Cary 100 Bio (Varian Inc., USA) spectrophotometer, at 565 nm *versus* a blank (prepared in the same way but without standard addition).

All assays were performed in triplicate and the results were expressed as mean±standard deviation (M±SD). The statistical interpretation of data was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

LACTUCA SATIVA ASSAY

Each solid extract (SYM, SEN, PET, TUSS) was dissolved in distilled water in order to obtain solutions with concentrations of 2.0, 1.5, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.005 mg/mL.

The experiment was conducted on *Lactuca sativa* seeds. 20 seeds per sample were placed on filter paper disks in Petri dishes of 90 mm diameter and treated with 5 mL of each solution. Distilled water was used as control [7].

The Petri dishes were kept in a Sanyo MLR-351 H plant growth chamber (San Diego, USA) at 25±1 ºC, 75±1% relative humidity and a photoperiod of 16:8 h (light: dark) [18]. Germination was monitored after 24h and the radicle length was measured using the application Image J version 1.46r (Wayne Rasband National Institutes of Health, USA). The values of root elongation were expressed in mm.

D'Agostino Pearson normality test ($\alpha = 0.5$) was performed in order to determine the normal distribution of root elongation values.

The inhibitory effect (Ef_i) of each extract, for all concentrations, was calculated with Microsoft Office Excel 2003 (Microsoft Corp., USA), using the following equation:

$$Ef_i = 100 - \frac{P}{M} \times 100 \quad (1)$$

where: P – average root elongation of the sample (mm), M – average root elongation of the control (mm), 100 – the results are expressed as a percentage.

Due to some abnormal distribution of the radicular elongation values, a non-parametric analysis (Kruskall Wallis test, post test Dunn) was performed.

Logarithm of concentration – inhibitory effect equations were obtained and the IC_{50} parameter (representing the concentration that inhibits the root elongation with 50%, compared to the negative control [8, 9]) was determined for each extract, using the GraphPad Prism v.5.0 software (GraphPad Software, USA).

RESULTS AND DISCUSSION

SPECTROPHOTOMETRIC ASSAY

A seven points linear calibration curve of senecionine in the 0.25 – 2 μg/mL range with good linearity ($R = 0.9990$) was obtained. The standard calibration curve and equation are presented in Fig. 2.

The highest content of PAs was found in SEN, followed by SYM. A much lower concentration of PAs was found in PET and TUSS, thus showing that the corresponding commercially available tea plant is less susceptible to induce hepatotoxicity.

The total PAs and the corresponding N-oxides content for the 4 plant extracts are summarized in Table 1.

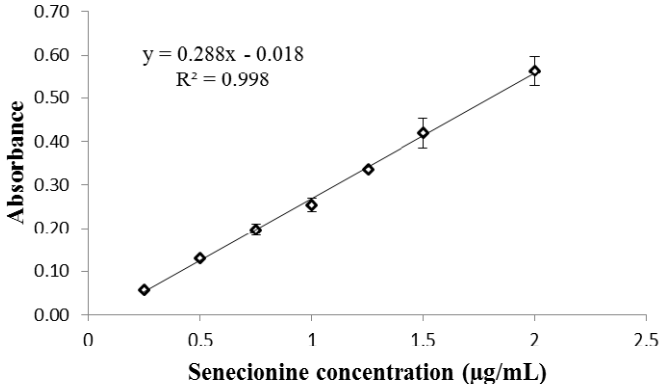

Fig. 2. The standard calibration curve and equation for senecionine.

Table 1

The total pyrrolizidine alkaloids and the corresponding N-oxides content (expressed as senecionine)

Sample	Total PAs (mg%)		N-oxides (mg%)		N-oxides (% of total PAs)
	M±SD, *n* = 3	**CI 95%**	**M±SD, *n* = 3**	***CI* 95%**	
SYM	150.24±10.35	124.53–175.95	36.15±2.89	28.96–43.34	24.06%
SEN	424.92±9.81	400.55–449.28	247.50±12.21	217.17–277.82	58.25%
PET	0.021±0.00091	0.018–0.023	0.013±0.008	0.011–0.015	60.64%
TUSS	0.0097±0.00072	0.008–0.012	0.0046±0.0003	0.0039–0.0053	47.09%

M – average; SD – standard deviation; *n* – assays number; *CI* – confidence interval.

The highest percentage of the N-oxide is found in PET, followed by the SEN, TUSS and SYM.

LACTUCA SATIVA ASSAY

All the extracts inhibited the root elongation of *L.sativa* compared to control. Root elongation values were abnormally distributed in the groups treated with TUSS (1.0, 0.5, 0.1 mg/mL), SEN (2.0 mg/mL), PET (1.0, 0.01, 0.005 mg/mL) and SYM (2.0, 1.0, 0.05, 0.01 mg/mL). For all the other tested groups, there was a Gaussian distribution of the data.

The results of Kruskal Wallis test highlighted the statistical significance for the inhibitory effect induced by all extracts at the tested concentrations compared to controls, as the probability of the null hypothesis was lower than 0.05 ($p < 0.0001$). The post-test Dunn showed that the inhibition of the growth is statistically significant at concentrations between 1.0 and 2.0 mg/mL for all

extracts, at 0.5 mg/mL for TUSS, SEN and SYM ($p < 0.05$), whereas concentrations within the range (0.005–0.1 mg/mL) had no effect on the root elongation ($p > 0.05$).

The tested concentration logarithms vs. inhibitory effect (%) curves are represented in Fig. 3.

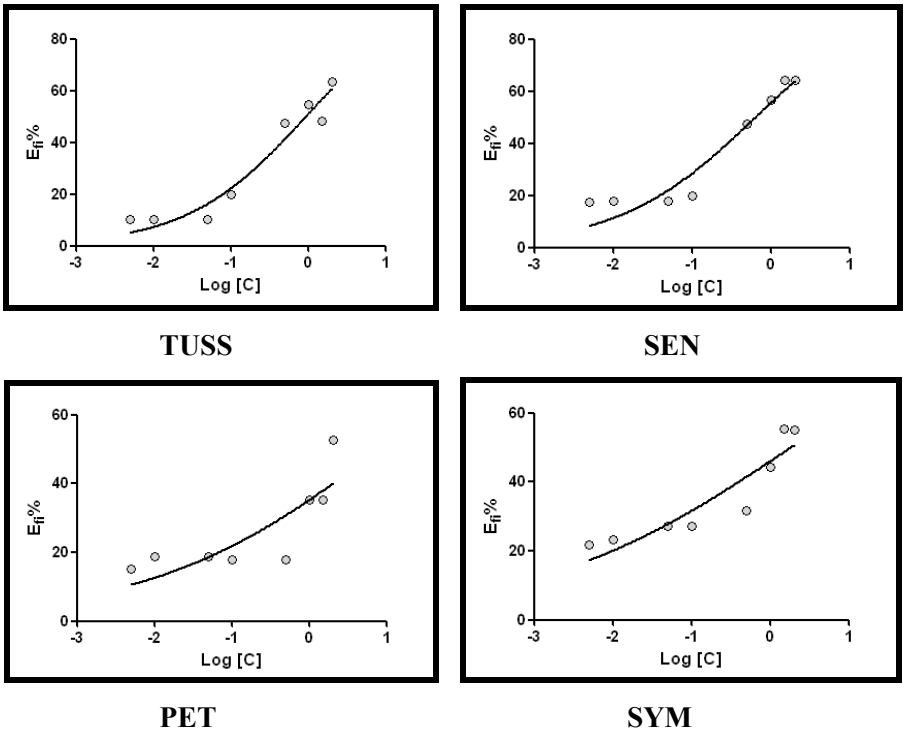

Fig. 3. The concentration-inhibitory effect curves for TUSS, SEN, PET and SYM.

Due to the absence of correlation between inhibitory effect and the tested concentrations, IC_{50} for PET could not be calculated. However, it can be assumed that the IC_{50} value for PET is between 1.5 and 2.0 mg/mL. For the other extracts the IC_{50} and confidence intervals values are presented in Table 2. SEN had the lowest IC_{50} value (0.62 mg/mL). This is about 50% less than TUSS and 2.6 times lower than PET and SYM. Thus, the most toxic extract was SEN, followed by TUSS, SYM and PET.

An interesting observation is that SEN (the extract with the highest concentration of PAs) exhibit the lowest IC_{50} and thus the highest toxicity. SYM, the extract with the second highest concentration of PAs, has the highest IC_{50}. This may be due to the presence in the extract, according to the literature, of allantoin [13], a substance that stimulates cell proliferation and organogenesis [11, 12].

Table 2

The assessment of phytotoxicity with *Lactuca sativa* bioassay

No. crt.	Sample	IC_{50}	95%*CI* of IC_{50}	Goodness of fit (r^2)
1	TUSS	0.91	0.56 – 1.49	0.9436
2	SEN	0.62	0.35 – 1.09	0.9298
3	PET	– [a]	– [b]	0.6651
4	SYM	1.64	0.53 – 6.08	0.8403

M – average, SD – standard deviation, *CI* – confidence interval; a – IC_{50} could not be calculated; the value is between 1.5 and 2.0 mg/mL; b – 95% *CI* is too wide and could not be calculated.

CONCLUSIONS

Pyrrolizidine alkaloids are toxic compounds found in certain plants. In this study, we have investigated if there is a correlation between the total pyrrolizidine alkaloids content of four plant species used as medicinal herbs (*Tussilago farfara, Symphytum officinale, Senecio vernalis* and *Petasites hybridus)* and their phytotoxicity, by means of *Lactuca sativa* root growth assay.

The highest content of PAs was found in *Senecio vernalis* extract (424.92±9.81 mg%), followed by *Symphytum officinale* extract (150.24±10.35 mg%) and *Petasites hybridus* extract (0.021±0.00091 mg%). The lowest concentration was found in *Tussilago farfara* extract (0.0097±0.00072 mg%).

The extracts inhibited the elongation of the *Lactuca sativa* radicle, in a dose-dependent manner. The IC_{50} could be computed only for the first three plant extracts. As shown by the IC_{50} index, the most toxic effect could be found in *Senecio vernalis* extract. An interesting observation was made upon the *Symphytum officinale* extract which, although having the second greatest concentration of PAs, showed the highest computable IC_{50}.

The results of our study showed that the analysed extracts have phytotoxic effects. However, further studies should be performed in order to determine whether the inhibitory effect is due to the PAs presence and how it is influenced by the other compounds of the extracts.

REFERENCES

1. COULOMBE, R.A., Pyrrolizidine alkaloids in foods, *Advances in Food and Nutrition Research,* 2003, **45**, 61–88.
2. LEBADA, R., A. SCHREIER, S. SCHERZ, C. RESCH, L. KRENN, B. KOPP, Quantitative analysis of the pyrrolizidine alkaloids senkirkine and senecionine in *Tussilago farfara* L. by capillary electrophoresis, *Phytochemical Analysis*, 2000, **11**, 366–369.
3. LOPEZ, G.D., J.P. CAMBERO, A.C. CALVO, Germination of *Lactuca sativa* seeds as pre-screening in toxicology studies in higher plants, *Toxicology Letters*, 2008, **180**, S 5, S182.

4. MATTOCKS, A.R., Spectrophotometric determination of unsaturated pyrrolizidine alkaloids, *Anal. Chem.*, 1967, **39**, 443–447.
5. MATTOCKS, A.R., Spectrophotometric determination of pyrrolizidine alkaloids – some improvements, *Anal. Chem.*, 1968, **40**, 1749–1750.
6. MAZOIR, N., A. BENHARREF, M. BAILÉN, M. REINA, A. GONZÁLEZ-COLOMA, Bioactive triterpene derivatives from latex of two *Euphorbia* species, *Phytochemistry*, 2008, **69**, 1328–1338.
7. MOITEIRO, C., M.J. MARCELO-CURTO, N. MOHAMED, M. BAILE´N, R. MARTINEZ-DIAZ, A. GONZALEZ-COLOMA, Biovalorization of friedelane triterpenes derived from the cork processing industry byproducts, *J. Agric. Food Chem*., 2006, **54**, 3566–3571.
8. NITULESCU, G.M., C. DRAGHICI, O.T. OLARU, New potential antitumor pyrazole derivatives: synthesis and cytotoxic evaluation, *Int. J. Mol. Sci*., 2013, **14**, 21805–21818.
9. OLARU, O.T., A.I. ANGHEL, V. ISTUDOR, R.V. ANCUCEANU, M. DINU, Contributions to the pharmacognostical and phytobiological study of *Fallopia aubertii* (L. Henry) Holub. (*Polygonaceae*), *Farmacia*, 2013, **61**, 991–999.
10. RAO, G.N., M.A. CORSON, B.C. BERK, Uric acid stimulates vascular smooth muscle cell proliferation by increasing platelet-derived growth factor A-chain expression, *The Journal of Biological Chemistry*, 1991, **266**, 8604–8608
11. ROEDER, E., Medicinal plants in Europe containing pyrrolizidine alkaloids, *Pharmazie*, 1995, **50**, 83–98.
12. SHETTY, K., Y. ASANO, K. OOSAWA, Stimulation of *in vitro* shoot organogenesis in *Glycine max* (Merrill.) by allantoin and amides, *Plant Science,* 1992, **81**, 245–251.
13. STAIGER, C., Comfrey: A clinical overview, *Phytother. Res*., 2012, **26**, 1441–1448.
14. YUET PING, K., I. DARAH, U.K. YUSUF, C. YENG, S. SASIDHARAN, Genotoxicity of *Euphorbia hirta*: An *Allium cepa* assay, *Molecules*, 2012, **17**, 7782 –7791.
15. ZASADA, I.A., W. KLASSEN, S.L. MEYER, M. CODALLO, A.A. ABDUL-BAKI, Velvetbean (*Mucuna pruriens*) extracts: impact on *Meloidogyne incognita* survival and on *Lycopersicon esculentum* and *Lactuca sativa* germination and growth, *Pest. Manag. Sci*., 2006, **62**, 1122–1127.
16. ****CHMP Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids*, (EMA/HMPC/893108/2011), 2012.
17. ****IPCS Environmental Health Criteria 80, Pyrrolizidine Alkaloids*, Geneva, 1988.
18. ****OECD Guidelines for Testing Chemical Products, Method 208, Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*, Paris, 2006.
19. ***Scientific Opinion on Pyrrolizidine alkaloids in food and feed, *EFSA Journal*, 2011, **9**, 2406–2540.