

Oral toxicity study of certain plant extracts containing pyrrolizidine alkaloids

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Abstract

Pyrrolizidine alkaloids (PAs) are a class of toxic compounds which are found in plants. Poisoning caused by these toxins is associated with acute and chronic liver damage. *Tussilago farfara* (coltsfoot), *Petasites hybridus* (common butterbur), *Senecio vernalis* (eastern groundsel) and *Symphytum officinale* (comfrey) are traditional phytotherapeutic species, which beside the therapeutic bioactive compounds contain PAs. The aim of the paper was to assess the safety of some dry extracts obtained from these species. For the determination of acute toxicity, Organization for Economic Cooperation and Development (OECD) Guideline No. 423 was used. For the determination of repeated dose oral toxicity, *Senecionis vernalis herba* and *Symphyti radix* extracts (250 mg/kg) were administered, by gavage, for 28 days, and their effects on animal weight, liver and biliary functions, hepatic tissue and oxidative stress were investigated. After the acute toxicity testing, the dry extracts were placed in the GHS Category V ($LD_{50} > 5000$ mg/kg, *p.o.*). For the subacute toxicity testing, no death or any signs of toxicity were observed. Also, no significant differences in biochemical parameters were observed between control and treated groups. The observed histopathological lesions were non-specific and were not consistent with the data reported in the literature for PAs exposure. In conclusion, the administration for 28 days, of the tested extracts, in a dose which correspond to a PAs concentration over the limits imposed in some countries, produced no hepatic and biliary toxic effects. Further studies, extended over a longer period of time, are needed in order to determine the safety of plant extracts containing PAs.

Keywords: pyrrolizidine alkaloids, hepatic toxicity, repeated dose toxicity, histopathological examination.

Introduction

Pyrrolizidine alkaloids (PAs) are a class of secondary metabolites synthesized exclusively by plants. Almost 400 PAs have been identified worldwide in more than 350 plant species and up to 13 families, mainly *Asteraceae*, *Boraginaceae* and *Fabaceae* [1].

The PAs usually contain a base (necine), a bicyclic five atoms ring that bears two or more hydroxyl groups that are esterified with necic acids (Figure 1). PAs are usually found as mixtures of the alkaloid base and their N-oxides (PANOs), which frequently predominate. The necines can be saturated or possess a double bond in the 1,2- position [2]. Only the group of PAs which structurally are 1,2-unsaturated is considered toxic [3].

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. Their hazard arises from the formation of pyrrolic metabolites

(dehydroalkaloids) in the liver able to bind to nucleophilic centers in tissues or cross-link DNA, leading to hepatotoxicity and carcinogenicity [4–7].

Numerous cases of liver cirrhosis with a high death rate were attributed to occasional or longer periods consumption of herbs containing PAs. These cases have been reported particularly in developing countries, where traditional medicine plays a very important [5].

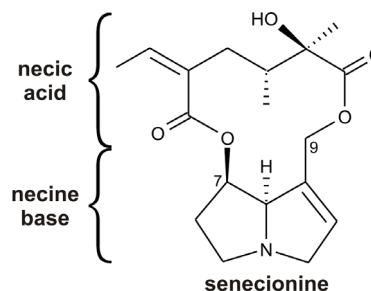


Figure 1 – Structural features of PAs.

Tussilago farfara (coltsfoot), *Petasites hybridus* (common

butterbur), *Senecio vernalis* (eastern groundsel) and *Symphytum officinale* (comfrey) are traditional phytotherapeutic species that, beside the therapeutic bioactive compounds, contain PAs. *Petasites hybridus* root is used mainly for the antispastic and anti-inflammatory effects in the treatment of migraine and dysmenorrhea [8, 9]. *Tussilago farfara* leaves are used to relief dry caught [10] and the aerial parts of *Senecio* species are used for their antidiarrheal, diuretic and expectorant properties [11]. *Symphytum officinale* roots are used in the treatment of stomach ulcers and other diseases of the digestive tract [12].

The aim of the paper was to assess the safety of some dry extracts obtained from these species. For this purpose, we investigated the oral toxicity after a single dose of *Symphyti radix* (SYM), *Petasitidis rhizoma* (PET), *Farfarae folium* (TUSS) and *Senecionis vernalis herba* (SEN) extracts. Also, a repeated dose toxicity study was conducted, using the extracts with the highest concentration of PAs: SYM and SEN extracts.

The extracts were obtained through a method, previously reported, that allows the extraction of large quantities of alkaloids and the total PAs content (including N-oxides) were measured using a spectrophotometric assay (Ehrlich's method), with senecionine as a standard substance. This method is specific for PAs with a double bound in the 1,2- position of the necine base, alkaloids known to be toxic. The highest content of PAs was found in SEN (424.92 ± 9.81 mg%), followed by SYM (150.24 ± 10.35 mg%), PET (2.11 ± 0.09 mg%) and TUSS (0.97 ± 0.07 mg%) [13].

Materials and Methods

Dry plant extracts

The obtaining of the dry extracts and their PAs content were previously reported [13]. Briefly, coltsfoot leaves, common butterbur roots teas and comfrey roots tea were purchased from retail stores. *S. vernalis* aerial part was harvested, the morphological characters of the vegetal material were compared with the ones quoted by literature [14] and conserved in laboratory conditions. The dried plants were grounded and refluxed twice for two hours with methanol acidified with citric acid to pH 2–3. The combined extracts were evaporated, under reduced pressure with a rotary evaporator system (Buchi, Switzerland) and atomized with a Mini Spray Dryer B-290 (Buchi, Switzerland).

Animals

The animals (mice and rats) were supplied by the rodent farm of “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania, and housed in plexiglas cages. Drinking water and food were provided *ad libitum* throughout the experiment. All animals were habituated for five days prior to the experiment to the testing environment and maintained on a 12-h light/dark cycle. The temperature and relative humidity were continuously monitored using an electronic hygro-thermometer. The temperature was between 21–24°C and the relative humidity was generally maintained at 35–45%.

All procedures were carried out in accordance with the Directive 2010/63/EU of 22 September 2010, regarding the protection of animals used for experimental and other scientific purposes [15].

Acute oral toxicity study

For the determination of acute toxicity, the acute toxic class method [Organization for Economic Co-operation and Development (OECD) Test Guideline No. 423] was used [16]. This method is a stepwise procedure that uses a small number of animals, which allows the placement of the substance within a Global Harmonised System (GHS) toxicity class, depending on the presence or absence of lethality of the animals.

Adult male NMRI mice (38.94 ± 3.17 g, $n=36$) were used. The animals were fasted four hours prior to treatment and two hours after with free access to drinking water.

A single dose of 2000 mg/kg body weight (bw) of each dry extract, 20% aqueous suspensions, was administered by gavage, to 12 mice (three mice/extract). After 48 hours, another 12 mice received the same treatment. Lack of lethality in previous stages of the research led to administration by gavage, of 5000 mg/kg bw of each dry extract, 50% aqueous suspensions.

All mice were observed in detail for any indications of toxicity effect within the first four hours after the treatment period, and daily further for a period of 14 days. The animals were weighed initially, 7 and 14 days after the start of the experiment. Visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

Repeated dose toxicity

Adult male Wistar rats (206.13 ± 35.04 g, $n=30$) were used. With one hour before and after the administering of the extracts, the food was removed. The animals were divided in three groups ($n=10$) and received daily 250 mg/kg bw by gavage of SEN or SYM extracts (25% aqueous suspensions). One group was used as control and received 1 mL/100 g distilled water. During the period of administration, the animals were observed closely, each day for signs of toxicity. Once a week, detailed clinical observations, outside the cage, was made for all animals: changes in skin, fur, eyes, mucous, occurrence of secretions and excretions and motor activity. The animals were weighed initially and several times throughout the experiment.

Biological tests

At the end of the 28 days, the animals were sacrificed and the blood was collected without anticoagulant. Biochemical measurements were performed on serum. Measurements were made using the spectrophotometric kits: LiquickCor – ALT (alanine transaminase), LiquickCor – AST (aspartate transaminase), LiquickCor – GGT (γ -glutamyltransferase) and LiquickCor – ALP (alkaline phosphatase) (Cormay, Poland).

The oxidative stress level was assessed by the measuring of TBARS (thiobarbituric acid reactive substances), using the spectrophotometric method with thiobarbituric

acid (TBA). TBARS react, at 100°C, with two molecules of thiobarbituric acid and form a chromophore (with maximum absorbance at 535 nm), product that is proportionally with the present TBARS [17].

Histopathological technique

After the slaughter, the liver was removed, weighed and examined macroscopically. The livers of five animals were preserved in 10% formalin solution, prepared by the histological technique of inclusion in paraffin and stained by the use of Hematoxylin–Eosin (HE) technique.

Statistical analysis

Results were statistically processed using Microsoft Excel 2010 and GraphPad Prism software and expressed as mean \pm standard deviation ($M \pm SD$). The Gaussian distribution was assessed by means of D'Agostino & Pearson test. Multiple group comparisons were performed using Student's *t*-test (for normal distribution) and Wilcoxon paired test and Mann–Whitney unpaired test (for abnormal distribution). The results were considered statistically significant for $p < 0.05$.

Results

Acute oral toxicity study

No toxic symptoms or mortality were observed in any animals. Skin, fur, eyes, mucous membrane, behavioral pattern, salivation and sleep pattern parameters of the treated animals were found to be normal. The body weight of all the mice increased after the administration of the extracts (Figure 2).

Repeated dose toxicity

No toxic symptoms, changes in appearance or behavior or mortality were observed in any animals treated for 28 days with 250 mg/kg bw of SEN or SYM extracts. A slight increase of body weight was observed for the groups treated with SEN and SYM extracts, when compared with the control (Figure 3).

After 28 days of SEN and SYM extracts administration, the animals were sacrificed and the serum was collected, in order to determine two parameters for hepatocellular injury: AST and ALT (Table 1), two parameters for hepatobiliary injury: GGT and ALP (Table 2) and a oxidative stress marker: TBARS (Table 3).

After the slaughter on the 28th day of the experiment, the liver was removed, weighed and examined macroscopically. There were no changes compared to the control group, in terms of color, appearance and texture of the harvested livers. There were no significant differences between the weights of the livers of the rats treated with the vegetable extracts when compared to the control group (Figure 4).

Histopathology revealed, in general, normal size hepatocytes structure and the absence of lipid droplets. In samples from all three groups, similar non-specific liver damage such as: degeneration of hepatocytes, discrete lymphocytic infiltrates, vascular hyperemia, vascular ectasia, were observed (Figures 5–7).

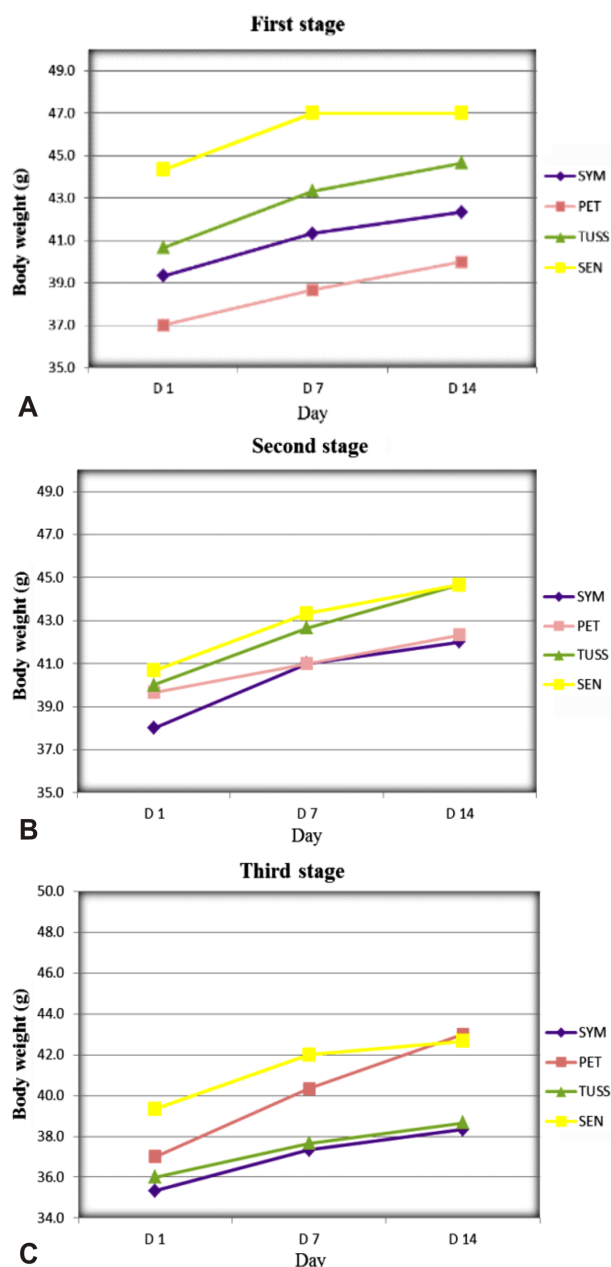


Figure 2 – Average change in body weight of animals treated by gavage, with the tested extracts: (A) Stage I – 2000 mg/kg bw; (B) Stage II – 2000 mg/kg bw; (C) Stage III – 5000 mg/kg bw. SYM: Symphyti radix; PET: Petasitidis rhizoma; TUSS: Farfarae folium; SEN: Senecionis vernalis herba.

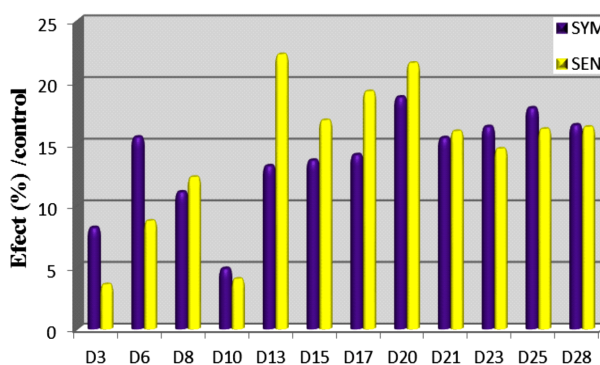


Figure 3 – The effect [%] of Senecionis vernalis herba (SEN) and Symphyti radix (SYM) extracts on body weight, compared with control.

Table 1 – Alterations of the hepatocellular injury parameters: AST and ALT for the animals in groups treated with SEN and SYM, compared with the control group (C)

Parameter	AST [U/L]			ALT [U/L]		
Group	C	SYM	SEN	C	SYM	SEN
M±SD	72.04±10.61	61.41±14.89	72.41±15.84	23.35±4.97	22.49±3.37	24.82±3.08
Distribution*	nd	nd	nd	nd	nd	nd
Effect [%]/control	–	-14.76	0.51	–	-3.68	6.30
Student's t-test (p)	–	ns	ns	–	ns	ns
		0.0567	0.9558		0.5861	0.4284

AST: Aspartate transaminase; ALT: Alanine transaminase; SYM: *Symphyti radix*; SEN: *Senecionis vernalis herba*; M: Mean; SD: Standard deviation; *According to D'Agostino & Pearson test; nd: Normal distribution; ns: Statistically non-significant ($p>0.05$).

Table 2 – Alterations of the hepatobiliary injury parameters: GGT and ALP for the animals in groups treated with SEN and SYM, compared with the control group (C)

Parameter	GGT [U/L]			ALP [U/L]		
Group	C	SYM	SEN	C	SYM	SEN
M±SD	1.17±0.47	1.08±0.39	1.08±0.39	113.65±13.2	107.13±12.97	108.32±27.36
Distribution*	nd	nd	nd	nd	nd	nd
Effect [%]/control	–	-7.69	-7.69	–	-5.74	-4.69
Student's t-test (p)	–	ns	ns	–	ns	ns
		0.6914	0.8996		0.1824	0.6740

GGT: γ -Glutamyl transferase; ALP: Alkaline phosphatase; SYM: *Symphyti radix*; SEN: *Senecionis vernalis herba*; M: Mean; SD: Standard deviation; *According to D'Agostino & Pearson test; nd: Normal distribution; ns: Statistically non-significant ($p>0.05$).

Table 3 – Oxidative stress marker TBARS for the animals in groups treated with SEN and SYM, compared with the control group (C)

Parameter	TBARS [nM]		
Group	C	SYM	SEN
M±SD	1.48±0.64	0.54±0.25	0.93±0.38
Distribution*	nd	nd	nd
Effect [%]/control	–	-63.58	-36.98
Student's t-test (p)	–	0.0010***	0.0470**

TBARS: Thiobarbituric acid reactive substances; SYM: *Symphyti radix*; SEN: *Senecionis vernalis herba*; M: Mean; SD: Standard deviation; *According to D'Agostino & Pearson test; nd: Normal distribution; ** $p<0.05$; *** $p<0.001$.

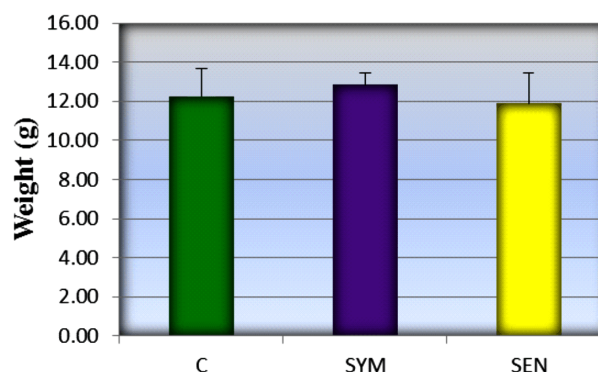


Figure 4 – Mean weight of liver tissue of the rats treated with *Senecionis vernalis herba* (SEN) and *Symphyti radix* (SYM) extracts and the control group (C).

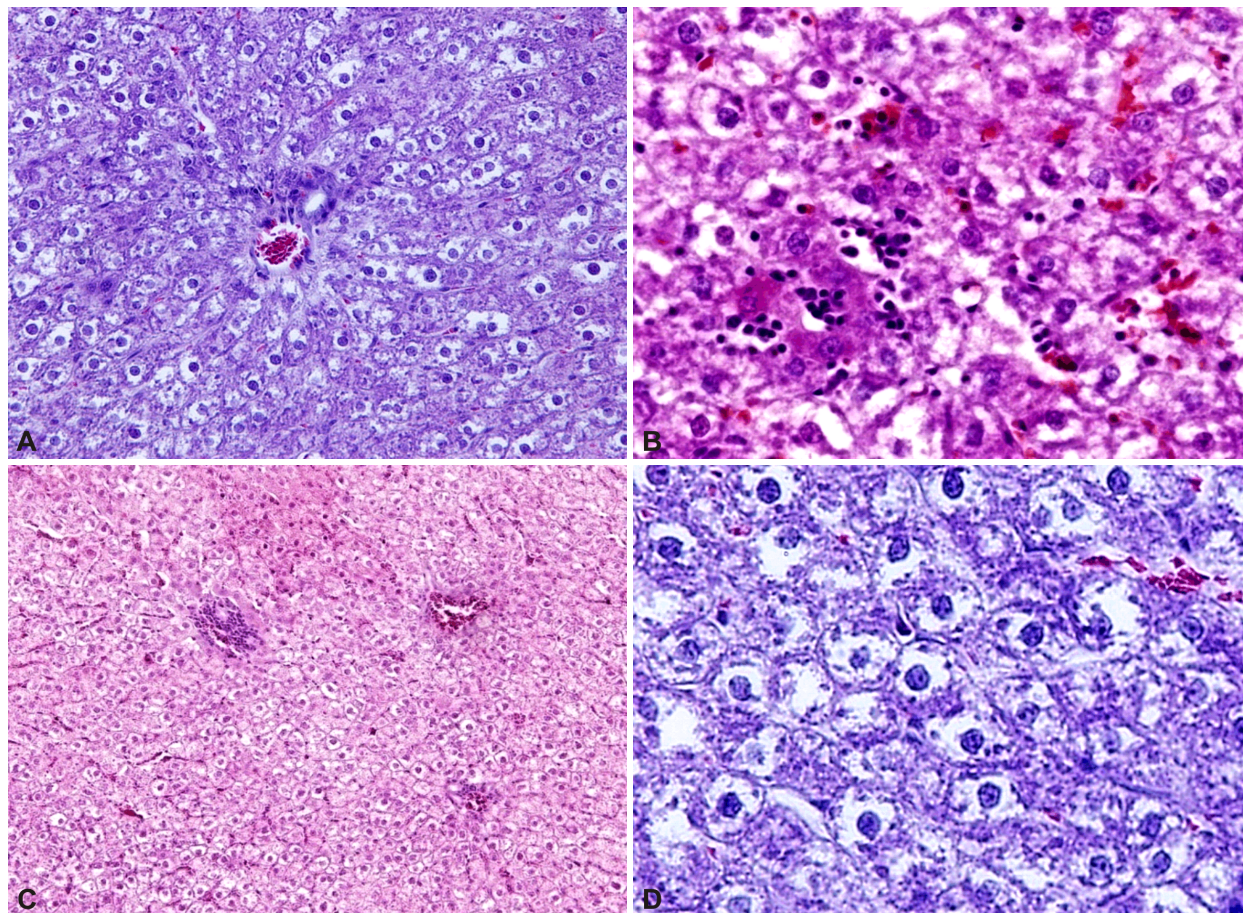


Figure 5 – Liver histopathology – *Symphyti radix* (SYM) 250 mg/kg bw, 28 days: (A) Hepatocyte degeneration; (B) Hepatocyte degeneration in different phases – very discreet lymphocyte infiltration; (C) Discreet perivascular mononuclear infiltrate; (D) Hepatocytes with normal appearance. HE staining: (A and C) $\times 100$; (B and D) $\times 400$.

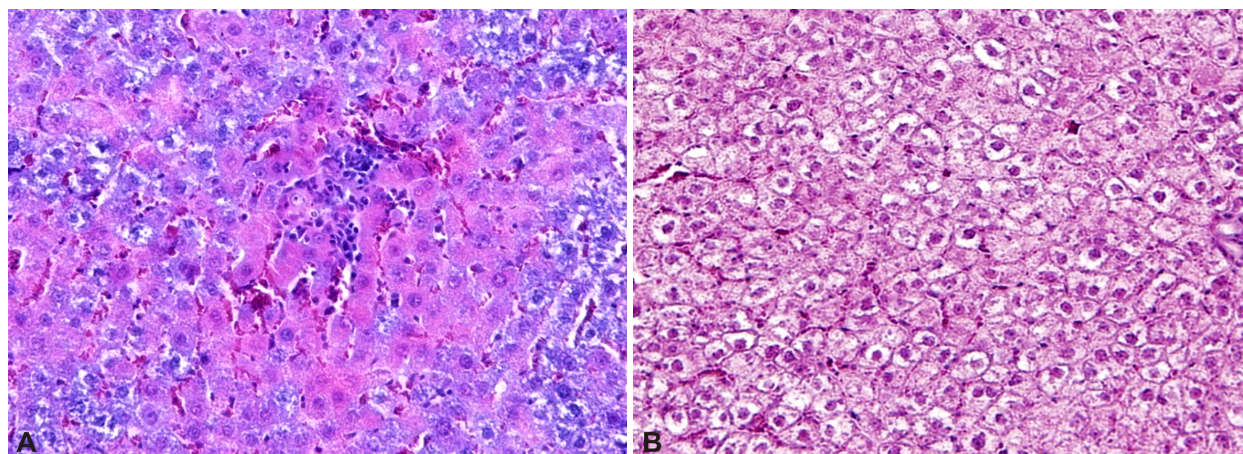


Figure 6 – Liver histopathology – *Symphyti radix* (SYM) 250 mg/kg bw, 28 days: (A) Discrete granular lymphocyte infiltration and hepatocyte degeneration; (B) Hepatocytes with normal appearance. HE staining, $\times 200$.

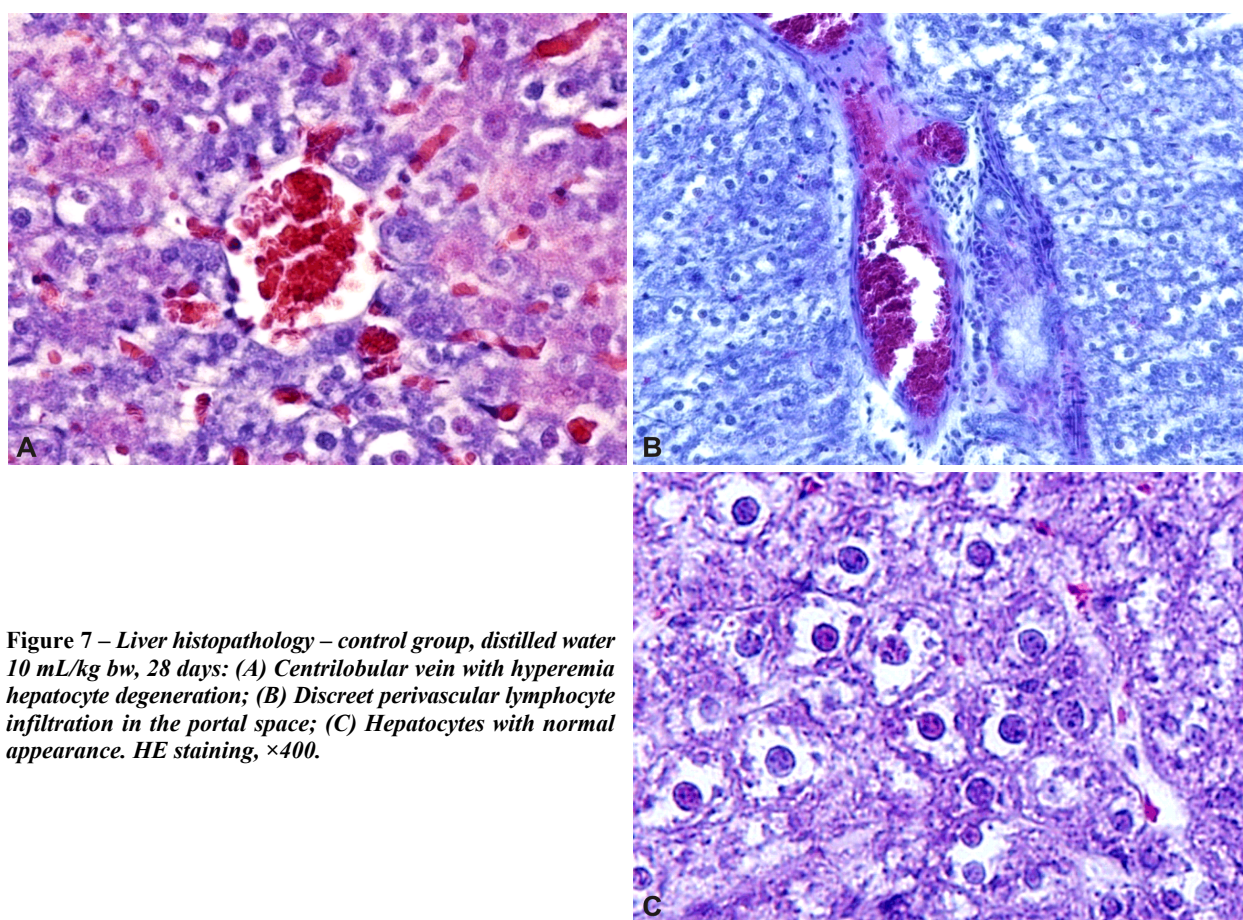


Figure 7 – Liver histopathology – control group, distilled water 10 mL/kg bw, 28 days: (A) Centrilobular vein with hyperemia hepatocyte degeneration; (B) Discrete perivascular lymphocyte infiltration in the portal space; (C) Hepatocytes with normal appearance. HE staining, $\times 400$.

Discussion

Acute oral toxicity study

The oral toxicity after a single dose was determined in accordance with the limit test described in the OECD Guideline No. 423 [16]. This test is performed, when available information suggests that mortality is unlikely to arise after a large initial dose. The test consists of administering a dose of 2000 mg/kg to a group of three animals. If no lethality is recorded, the test is repeated using the same dose and same number of animals. If no lethality is recorded again, a dose of 5000 mg/kg can be given to a group of three animals.

Because of the lack of toxicity and mortality at doses of 2000 mg/kg bw and 5000 mg/kg bw, the dry extracts SEN, SYM, PET and TUSS were placed in the GHS Category V ($LD_{50} > 5000$ mg/kg, *p.o.*), according to data contained in the Globally Harmonized System [18].

Repeated dose toxicity

By administering the extracts for 28 days, information about possible health hazards that may arise from repeated exposure over a relatively limited period of time can be found [19].

Due to the higher concentration of PAs, SEN and SYM extracts were selected for the determination of

repeated dose toxicity. Each extract was administrated by gavage (250 mg/kg), daily for 28 days. This dose corresponds to 1/20 of the determined LD₅₀, and to a total PAs (with double bond in the 1,2- position of the heterocycle, including the N-oxides) content of approximately 1.06 mg/kg bw for SEN and 0.375 mg/kg for SYM. In European countries (Germany, Switzerland), for phytotherapeutic products that contain PAs with double bond, a maximum daily dose of less than 1 µg is regulated [2]. The administered doses of extracts, exceed by far this limit.

The administration of the tested extracts produced a slight increase of body weight, for the treated groups, when compared with the control, which may indicate their lack of toxicity.

Biochemical parameters

Knowing the hepatotoxic potential of PAs, the effects the extracts have on liver and biliary function were investigated. According to the European Medicines Agency (EMA) Guidelines, on non-clinical evaluation of drug-induced liver injury the panel for hepatocellular injury should include at least two of the following: ALT, AST, sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GDH). Similarly, at least two of the following serum parameters for identification of hepatobiliary injury should be measured: ALP, GGT, 5'-nucleotidase (5'-NT) and total bilirubin (TBILI) [20].

After the slaughter of the animals, at the end of the 28 days, AST and ALT were determined for the assessment of the hepatocellular injury, and GGT and ALP for the hepatobiliary injury. The biochemical parameters of the animals from groups treated with SEN and SYM extracts, showed minor changes (statistically non-significant), when compared with controls.

Numerous studies have demonstrated the involvement of oxidative stress in hepatotoxicity, due to the exposure to certain substances like: alcohol, paracetamol, carbon tetrachloride, etc.

Liang *et al.* (2009) evaluated the oxidative stress induced by pyrrolizidine alkaloid clivorine on liver cell cultures L-02. Total cellular antioxidant capacity, glutathione-S-transferase (GST) and glutathione reductase (GR) were determined. An increase in the total cellular antioxidant capacity and a drop in GTS and GR were observed. It has also been observed that the antioxidant substances like ascorbic acid, vitamin E derivatives, mannitol and dithiothreitol, prevent the signs of cytotoxicity due to clivorine. These results suggest that the pyrrolizidine alkaloid produces oxidative stress-mediated cell damage [21]. Also, Liu *et al.* (2010) demonstrated that isoline (a PA extracted from *Ligularia duciformis*) induces oxidative stress characteristic lesions on multiple mice organs, with the liver being the most sensitive organ [22].

Moreover, PAs active metabolites bound to glutathione, an antioxidant substance, the body is unable to detoxify reactive oxygen species and thus leading to increased oxidative stress and indirectly membrane peroxidation [23]. Starting from these premises, we decided to determine if the administration of SEN and SYM extracts could induce oxidative.

Experimental results showed a decrease in serum TBARS, compared with the control. The decrease was

statistically significant both for the group treated with SYM (-63.58%; $p=0.0010$) and for the group treated with SEN (-36.98%; $p=0.047$). This reduction can be attributed to other active ingredients of the plant extracts. Thus, the literature reports the antioxidant effect of *Symphytum officinale*. Neagu *et al.* (2010) demonstrated the antioxidant effect of some extracts obtained from *Symphyti radix* by the use of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods [24]. Similarly, Puertas-Mejia *et al.* (2012) have found that the antioxidant effect of the extract obtained from the leaves of comfrey is comparable to that of ascorbic acid [25].

Histopathological technique

According to EMA Guideline on non-clinical evaluation of drug-induced liver injury, the correlation of animal/human toxicity may be improved by incorporation of animal histopathology data [20].

The weight of the livers taken from the rats from the groups treated with vegetable extracts compared to the control group showed no significant changes (4.84% SYM and -3.02% for SEN). These results are not consistent with the literature, which reports hepatomegaly as one of the main symptoms of intoxication in acute phase, with high doses of alkaloids [26] or the shrinking of the liver after the consumption of lower doses of PAs for a longer period of time [27].

Histopathological lesions observed for the groups treated with SYM and SEN extracts and the control group are non-specific and do not coincide with those reported in the literature. The specific histopathological changes that occur after PAs ingestion is megalocytosis [4, 26, 28–30]. Other changes characteristic of this type of poisoning are intrahepatic blood vessels fibrosis, bile duct hyperplasia, connective tissue growth, mild steatosis [26, 30–32].

Conclusions

All the tested extracts were placed in the Category V of acute oral toxicity, which is considered practically non-toxic. The administration for 28 days, of SEN and SYM extracts, 250 mg/kg bw, by gavage, dose corresponding to a PAs concentration over the limits imposed in some countries, produced no hepatic and biliary toxic effects. Further studies, extended over a longer period of time, are needed in order to determine the safety of plant extracts containing PAs and how it is influenced by other components of the extracts.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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