SUMMARY OF THE PhD THESIS

Experimental model for *in vitro* and *in vivo* biological efficacy and toxicological evaluation of plant extracts

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INTRODUCTION

For ages, both humans and animals have been instinctively using plants for prevention and treatment of various diseases. In recent decades, due to the rapid spread of antimicrobial and anthelmintic resistance, the interest in herbal medicines (HMs) and traditional medicine has increased tremendously and led to the start of the "Return to Nature" trend (KHAN and AHMAD, 2019). Currently the scientific literature is based on a small number of books on complementary medicine and veterinary phytotherapy, and clinical *in vivo* studies regarding the use of phytopharmaceutical remedies in animals are limited. However, for integrating herbal medicine into evidence-based medicine, *in vitro* and *in vivo* studies on the biological effects, chemical composition, pharmacokinetics of secondary metabolites and toxic potential of plants are requisite. Currently, it is certain that studying the bioactive potential of plants and plant extracts is still at the beginning of the road, one of the problems faced by researchers in the field of phytotherapy being the poor replicability of studies. An important cause is the lack of information regarding the chemical composition of the plant extracts used, the extraction method and the administered dose. Also, the biochemical profile of the plants may vary significantly within the same species depending on the particularities of the surrounding environment. At the same time, globally there are major differences between countries in toxicity testing protocols, mainly due to the rapid development of research and the emergence of new testing methods. For this reason, it is currently necessary to establish complex protocols, *in vitro* and *in vivo*, to determine the biological effects, mechanisms of action, acute and chronic toxic potential of plant extracts.

WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

Currently, 25% of modern medicines are plant-derived, medicinal herbs and their bioactive compounds are regarded as safer and healthier substitutes to the synthetic drugs, especially in long-term use (KHAN şi AHMAD, 2019). However, it was estimated that only 15% of species have been studied for their chemical composition, and around 6% for their therapeutic effects (ATANASOV şi colab., 2015). **The aim** of the research was to demonstrate the therapeutic effects known in Romanian traditional medicine and the reduced toxic potential of several species of medicinal plants existing in the spontaneous flora of Romania, through their complex *in vitro* and *in vivo* evaluation.

The general objective of the thesis was the selection, through eliminations carried out in each phase of the research, of a plant used in ethnomedicine and the determination of its chemical composition, evaluation of therapeutic effect and *in vitro* and *in vivo* toxic potential.

In order to achieve the formulated main objective, we have outlined **3 specific objectives** as follows:

- Evaluation of the *in vitro* anthelmintic activity of six plants commonly used in traditional Romanian medicine for the treatment of parasitic diseases in both humans and domestic animals: *Achillea millefolium*, *Artemisia absinthium*,

Centaurium erythraea, *Gentiana asclepiadea*, *Inula helenium*, and *Tanacetum vulgare*;

- Determination of the chemical composition, *in vitro* antioxidant, antibacterial and cytotoxic potential of two medicinal plant species selected based on the results obtained in the first phase of the research;
- *In vivo* investigation of the safety and potential dose-dependent toxic effects of the extract obtained from the medicinal plant selected in the second phase of the research, using the murine experimental model.

In order to achieve the proposed objectives, the present research was divided into **4 distinct studies**:

S1 ***In vitro*** **evaluation of the anthelmintic effect of six medicinal plants from the spontaneous flora of Romania by means of the egg hatching assay (EHA) and the larval development assay (LDA):**

- ✓ Determination of the *in vitro* ovicidal potential of aqueous extracts using the egg hatching assay (EHA);
- ✓ Determination of the *in vitro* larvicidal potential of the tested extracts, through the larval development assay (LDA);
- ✓ Statistical analysis of the obtained results and the determination of median lethal concentration (LC_{50}).

S2 **Chemical characterization by liquid chromatography - mass spectrometry analysis (LC/MS) and *in vitro* evaluation of antioxidant and antibacterial effect of *I. helenium* and *G. asclepiadea***:

- ✓ Quantification of total phenolic, flavonoid, and phenolic acids content by means of the spectrophotometric methods;
- ✓ Identification and quantification of plants' chemical constituents by LC/MS analysis (liquid chromatography - mass spectrometry);
- ✓ Determination of the potential antioxidant effect of the tested extracts using the FRAP and DPPH methods;
- ✓ Evaluation of antimicrobial efficacy of tested extracts on six reference strains: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29219, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 and *Salmonella enteritidis* ATCC 13076.

S3 ***In vitro*** **evaluation of the cytotoxic potential of medicinal plants included in the second study, on rat intestinal epithelial cells**:

- ✓ The preparation of rat intestinal epithelial cell culture;
- ✓ Evaluation of cell viability by using MTT assay;
- ✓ Determination by statistical methods of the correlation between the main classes of analyzed chemical compounds (polyphenols, flavonoids

and total phenolic acids) and the cytotoxic effect of the ethanolic extracts included in the study.

S4 ***In vivo*** **investigation of the safety and potential dose-dependent toxic effects of the extract obtained from *I. helenium*, plant selected following the analysis of the results obtained in studies 2 and 3, using the murine experimental model:**

- ✓ Performing the acute toxicity test by the Up and Down method and determining the median lethal dose (LD_{50}) by statistical methods;
- ✓ Adaptation of a complex *in vivo* subacute (28 days) and subchronic (56 days) toxicity testing protocol;
- ✓ Evaluation of the impact on the digestive system by determining the growth parameters and the structure of the intestinal bacterial population;
- ✓ Assessment of the systemic toxic potential of the aqueous extract of *I. helenium*, by means of histological, immunohistochemical, hematological and biochemical examinations.

STRUCTURE OF THE DOCTORAL THESIS

The doctoral thesis entitled „*Experimental model for in vitro and in vivo biological efficacy and toxicological evaluation of plant extracts*" is structured in two parts: Part I - Current state of knowledge and Part II - Personal contribution, comprising a total of 162 pages, 43 figures, 28 tables and 274 references.

PART I - Current state of knowledge

First part of the doctoral thesis is structured in 2 chapters and comprises 30 pages. **Chapter I** is entitled "Interaction and coexistence between "parasitome", microbial communities and host" and includes 3 subchapters, which summarize information on the direct and indirect interaction mechanisms between the microbiome and the intestinal "parasitome", as well as their importance for the animal organism. **Chapter II** is entitled "Plants used in veterinary anthelmintic therapy: from ethnomedicine to the modern scientific therapy" and includes 1 subchapter, in which are presented notions related to the phytotherapy and plant bioactive compounds, but also the mechanism of action of the main classes of the chemical compounds with anthelmintic activity. Additionally, the chapter contains a synthesis of the existing data regarding the uses in ethnomedicine, the biological activities and the chemical composition of seven species of medicinal plants with anthelmintic potential existing in the spontaneous flora of Romania: *Cichorium intybus* L., *Inula helenium* L., *Gentiana asclepiadea* L., *Achillea millefolium* L. s.l., *Artemisia absinthium* L., *Tanacetum vulgare* L. and *Centaurium erythraea* Rafn.

PART II - Personal contribution

Part II is structured in 8 chapters and has a number of 84 pages. In this part are presented the working hypothesis and general objectives of the research, the materials and methods used, the results of the studies carried out, the general conclusions, the recommendations formulated on the basis of the obtained results, the originality and the innovative contributions of the thesis.

Chapter III is entitled "Working hypothesis and research objectives" and describes the working hypothesis, the aim, the general objective and the specific objectives of the thesis.

Chapter IV is named "Materials and methods – general aspects" and briefly presents the way the investigations are organized, the plant, biological material and chemical compounds used in the research, as well as the statistical analysis methods applied.

Chapter V presents **Study I** entitled "*In vitro* determination of the anthelmintic effect of several plant extracts against *Equus asinus* intestinal strongyle". This study evaluated the ovicidal and larvicidal effects of 6 aqueous extracts of *I. helenium*, *G. asclepiadea*, *A. millefolium*, *A. absinthium*, *T. vulgare* and *C. erythraea*, on intestinal nematodes isolated from the species *Equus asinus*. The 6 species of plants included in the study are found in the composition of herbal anthelmintics (teas, capsules, tablets), sold in Romania. Also, 200 third stage strongyle larvae L3 were morphologically identified and classified into the two subfamilies: Cyathostominae and Strongylinae.

Chapter VI presents **Study II** entitled "Phytochemical characterisation of *Inula helenium* and *Gentiana asclepiadea* extracts", the purpose of which was to determine the chemical composition and potential antioxidant and antibacterial effect of the ethanolic extracts. The two species, *I. helenium* and *G. asclepiadea*, were selected for this study based on the results obtained in Study I. The total amount of polyphenols, flavonoids and phenolic acids was determined by spectrophotometric methods, and by the LC/MS method the compounds were identified and quantified plants' chemical compounds. FRAP and DPPH methods were used to determine the antioxidant effect of the ethanolic extracts. The evaluation of the antimicrobial action was carried out by the agar well-diffusion assay and broth microdilution assay, subsequently calculating MIC, MBC and the MIC index. Through the statistical analysis of the obtained data, the correlation between the main classes of chemical compounds analyzed (polyphenols, flavonoids and total phenolic acids) and the antioxidant and antibacterial effect of the extracts was determined.

Chapter VII presents **Study III** entitled "*In vitro* effect of *Inula helenium* and *Gentiana asclepiadea* ethanolic extracts on enterocytes' viability". The aim of this study was to determine the potential toxic effect of the ethanolic extracts of *I. helenium* and *G. asclepiadea* on enterocytes, which come into direct contact with the extract, the

intestinal lumen being the place where the extracts act on parasites. The MTT assay was used to determine cell viability, and through statistical data processing, the value of the half-maximal inhibitory concentration (IC_{50}) was calculated. Additionally, the correlation between the classes of chemical compounds analyzed in Study II and the cytotoxic effect of the extracts was determined.

Chapter VIII presents **Study IV** entitled "Acute, subacute and subchronic toxicity study – Experimental model". The aim of this study was to develop a complex protocol for ante- and postmortem assessment of the *in vivo* toxic potential of plant extracts on the body. The species *I. helenium* was selected following eliminations carried out at each stage of the research, but also based on bibliographic research. The root of *I. helenium* has an anthelmintic, selective antibacterial, antioxidant and prebiotic effect, respectively we consider that it has the ability to modulate the intestinal biome. This chapter provides information on the impact of oral administration of the extract, in a single dose (acute toxicity) or repeated doses (subacute and subchronic toxicity), on the animal's behavior, total blood count, growth parameters, intestinal bacterial population, digestive and excretory system.

Chapter IX is titled "General conclusions and recommendations" and includes the main conclusions and recommendations derived from 4 studies conducted in this research. **Chapter X** is titled "Originality and innovative contributions of the thesis", representing the synthesis of the main elements of originality of the thesis and the contribution to the development of a complex protocol for *in vitro* and *in vivo* testing of medicinal plants.

RESEARCH RESULTS

Chapter V (Study I).

Egg hatching assay (EHA). Aqueous extract of *I.. helenium* showed the most significant effects ($p<0.01$), inhibiting egg hatching at concentrations between 125 and 0.976 mg/mL, and the lowest LC_{50} value of 0.041 mg/mL (95% CI 0.01-0.16). *A. absinthium* exhibited strong inhibitory egg hatching effect ($p<0.01$) at concentrations between 125 and 3.9 mg/mL, with LC_{50} value of 0.486 mg/mL (95% CI 0.21-1.09). Followed in decreasing order by the aqueous extracts of *A. millefolium*, *T. vulgare* and *G. asclepiadea. C. erythraea* showed low ovicidal effect and did not exhibit a concentration-dependent pattern.

Larval development assay (LDA). *G. asclepiadea* and *I. helenium* aqueous extracts showed the most potent inhibitory effects in terms of larval development inhibition at concentrations between 125 and 0.976 mg/mL, having LC_{50} values of 0.36 (95% CI 0.19-0.72) and 0.41 (95% CI 0.27-0.62) mg/mL, respectively. Followed in decreasing order by the aqueous extracts of *A. millefolium*, *T. vulgare* and *A. absinthium*. The weakest anthelmintic activity against L3 nematodes was exhibited by *C. erythraea*.

Identification of larvae. Cyathostomin species represented 74% of the total L3 larvae number. *Cyathostomum sensu latum* type A (27%), type C (20%) and *Trichostrongilus axei* (10%) were the most frequent. Five species (26% of all species recovered) belonged to Strongylinae subfamily.

Chapter VI (Study II).

Total phenolic (TPC), flavonoid (TFC) and phenolic acids (TPA) content. Values obtained for TPC, TFC and TPA assays were significantly higher ($p < 0.05$) in the *I. helenium* ethanolic extract compared to the one obtained from *G. asclepiadea* (Table 1). The difference of TPC between *I. helenium* and *G. asclepiadea* was further confirmed by variations in the concentrations of caffeic and chlorogenic acids, identified and quantified by LC/MS analysis in both tested extracts.

Tabel 1

Total polyphenolic (TPC), flavonoid (TFC), and phenolic acids (TPA) content of Gentiana asclepiadea and Inula helenium extracts

Sample	TPC (g GAE/100 g Dry Plant Material)	TFC (g RE/100 g Dry Plant Material)	TPA (g CAE/100 g Dry Plant Material)
G. asclepiadea	2.144 * ± 0.088	0.280 * ± 0.014	0.224 * ± 0.030
I. helenium	3.066 * ± 0.041	0.602 * ± 0.016	1.182 * ± 0.017

Each value represents the mean ± standard deviations of three independent measurements. GAE: Gallic acid equivalents; RE: rutin equivalents, CAE: caffeic acid equivalents. * $p < 0.05$ G. asclepiadea vs. I. helenium.

LC/MS identified chemical compounds. Major compounds identified in *I. helenium* roots by the LC/MS analysis of the extract were caffeic acid (234.0 ± 2.1 µg/g dry plant material), chlorogenic acid (2284.1 ± 11 µg/g dry plant material), chrysin, luteolin, and hesperetin. *I. helenium* extract was also found to contain luteolin-*7-O*-glucoside and naringenin. Major compounds identified in *G. asclepiadea* roots were amarogentin (27.8 ± 0.3 µg/g dry plant material), apigenin, luteolin-*7-O*-glucoside, naringenin, and rutoside. In addition to the above-mentioned compounds, *G. asclepiadea* root extract contained *trans-p*-coumaric acid (192.8 ± 1.0 µg/g dry plant material), caffeic acid (169 ± 1.2 µg/g dry plant material), chlorogenic acid, luteolin, and unquantifiable amounts of salicylic acid, chrysin, and quercetin. The presence of amarogentin in *G. asclepiadea* roots was previously reported by Szucs et al. (2002), but only in trace amounts. Identification and quantification of amarogentin in *G. asclepiadea* harvested from Romania, is of scientific interest, since the population of other gentian species of high medicinal interest is decreasing worldwide (*G. lutea*, *G. punctata*, *G. dinarica*), positioning it as a promising substitute.

Antioxidant capacity. Based on the obtained results, both ethanolic extracts presented significant antioxidant capacities (Table 2), and this potential was significantly ($p < 0.05$) higher in case of *I. helenium* compared to that of *G. asclepiadea*. Coeficientul Pearson coefficients established for FRAP and TPC, TFC, and TPA content ($r^2 = 0.999$, $r^2 = 0.996$, and $r^2 = 0.999$, respectively, $p < 0.05$) and negative correlation between DPPH values and the above-mentioned compounds ($r^2 = -0.999$, $r^2 = -0.997$,

and r^2 =- 0.999, respectively, $p < 0.05$), confirm that antioxidant activity of plant extracts depends on their TPC, TFC and TPA content.

Table 2

Antioxidant capacity of G. asclepiadea and I. helenium root ethanolic extracts

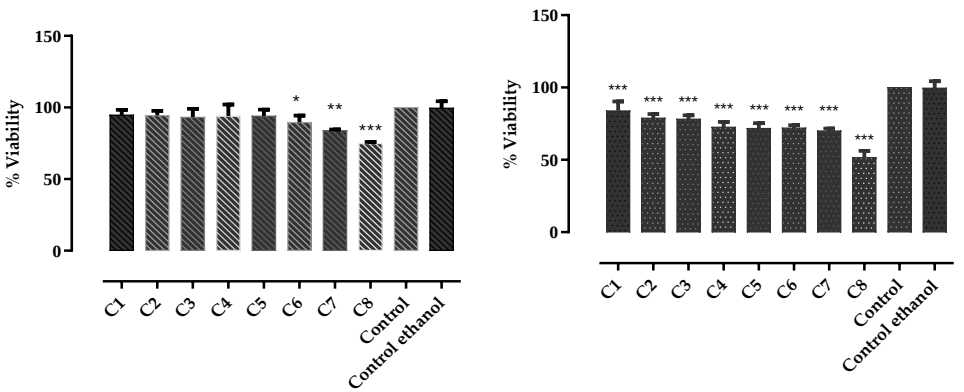
Sample	FRAP (µmol TE/ 100 mL de Extract)	DPPH, IC_{50} (µg/mL)
I. helenium	629.04 * ± 2.07	173.2 * ± 3.40
G. asclepiadea	145.23 * ± 3.60	363.7 * ± 0.89

Values represent the mean ± standard deviations of three independent measurements, *$p < 0.05$ *G. asclepiadea* vs. *I. helenium.* FRAP – ferric reducing antioxidant power; DPPH - 2,2-diphenyl-1-picrylhydrazyl

Antibacterial activity. The potency of the antimicrobial efficacy of both tested extracts varied depending mostly on the bacterial type, with a more intense inhibitory effect expressed against the Gram-positive species (*Bacillus cereus* > *Staphylococcus aureus* > *Enterococcus faecalis*), compared to the Gram-negative. Values obtained for the inhibition zone diameter were significantly lower in the case of *G. asclepiadea* and *I. helenium* root extracts ($p < 0.05$) compared to those of gentamicin. *S. aureus* and *B. cereus* showed higher susceptibility when exposed to a combination of the two extracts, obtained values being similar to those induced by gentamicin ($p > 0.05$). The bactericidal efficacy was clearly pointed out against all tested bacterial species (MBC/MIC ≤ 4).

Chapter VII (Study III).

G. asclepiadea roots (Fig. 1a) did not exhibit any cytotoxic activity at the tested concentrations (0.0079–0.4726 µmol GAE/mL), cell viability ranging from 94.83 ± 3.58 to 74.89 ± 0.97%, respectively.



(**a**) (**b**)

Fig. 1 Inhibitory effects of (a) *G. asclepiadea* and (b) *I. helenium* root extracts on rat intestinal epithelial cells, at eight different concentrations C1–C8 calculated according to the TPC (µmol GAE/mL extract): ranging between 0.0079 and 0.4786 for *G. asclepiadea* extract, and from 0.0135 to 0.8068 for *I. helenium* extract. Control—untreated cells, Control ethanol—cells treated with ethanol. Values are represented as mean ± SD. Statistically significant differences between treated and untreated cells (control): * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$

The IC_{50} dose for *G. asclepiadea* extract was 1.1097 ± 0.028 µmol GAE/mL. A strong linear correlation was observed between the cell viability and the TPC of extract.

At concentrations between 0.0135 and 0.5379 µmol GAE/mL, the ethanolic extract of *I. helenium* roots did not exhibit any cytotoxic activity, the cell viability ranging from 83.92 ± 6.37 to 70.11 ± 1.55% (Fig. 1b). However, at a concentration of 0.8068 µmol GAE/mL, a mild cytotoxic activity was observed, cell viability being 51.54 ± 4.68%. The IC_{50} dose of *I. helenium* extract was 0.9093 ± 0.016 µmol GAE/mL. Similar to the *G. asclepiadea* extract, a strong linear correlation was noticed between the cytotoxic effect of *I. helenium* extract and its TPC (r^2 = 0.9485).

Chapter VIII (Study IV).

Acute toxicity - Real-time RGS. In the acute toxicity study performed using Up and Down method, none of the animals showed signs of toxicity at any of the tested doses (175 – 5000 mg/kg). In result, the median lethal dose for the aqueous extract of *I. helenium* could not be calculated and was considered higher than 5000 mg/kg, extract being classified as practically nontoxic substance.

Subacute and subchronic toxicity. Animals (n = 24, 12 females și 12 males) were divided into 3 groups (4 females and 4 males each): Group 1 - control, Group 2 - received a dose of 300 mg/kg of extract, Group 3 - 600 mg/kg. Aqueous extract of *I. helenium* was administered daily, once a day, by gavage, during 28 days (subacute toxicity), on day 29th the interim sacrifice was performed, being sacrificed 4 animals (2 females and 2 males) from each group. The remaining rats continued to receive the extract until day 56 of the study (subchronic toxicity). On the 57th day of the study, the terminal sacrifice was performed.

Body weight gain and feed conversion ratio. Although the values of body weigh gain (Fig. 2) and feed conversion ratio varied throughout the 8 weeks of the study period, the values were within the physiological range and variations were not considered as treatment or dose-related.

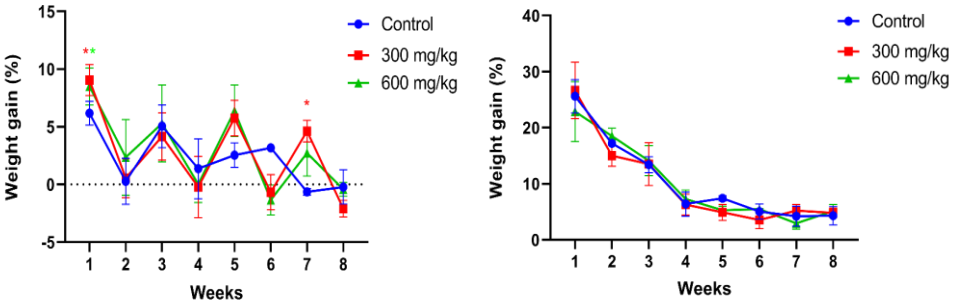


Fig. 2 Weekly body weight gain of a) females și b) males treated with various doses of aqueous extract of *I. helenium*. Data with asterisk are significantly different compared with the group 1 ($p \leq 0.05$)

Intestinal bacterial population. Using Vitek®2 – Compact 15 system the following species were identified: *Escherichia coli*, *Staphylococcus lentus*, *Enterococcus faecium* and *Proteus mirabilis*. Isolated bacteria are a part of the normal intestinal bacterial population of rats (AL-NAJJAR et al., 2021).

Necropsy examination and relative organ weight (ROW). Necropsy examination performed at interim and terminal sacrifices revealed no pathological findings at the level of digestive, excretory, respiratory, circulatory, lymphatic, or reproduction systems. Statistical analysis of lungs, heart, liver, and kidneys' ROW revealed no significant differences between groups, at any of the tested doses.

Histological findings. Examination of the samples stained using Goldner's Trichrome coloration method, revealed the absence of histological lesions at the level of gastric, small intestine and colonic mucosa (Fig. 3).

Fig. 3 Histological appearance (TG coloration) of: A) gastric mucosa (group 3, subacute toxicity) - gastric pits (C) and fundic glands (G, arrow) with no structural modifications; B) small intestine mucosa (group 2, subacute toxicity) - nomal aspect of enterocytes (black arrowhead) and intestinal glands (yellow arrowhead), absence of quantitative and qualitative changes of the cell infiltration in the lamina propria (arrow); C) colonic mucosa (group 3, subchronic toxicity) - absence of microscopic lesions at the level of surface epithelium (black arrowhead), glandular epithelium (yellow arrowhead) and normal levels of infiltrate in the lamina propria

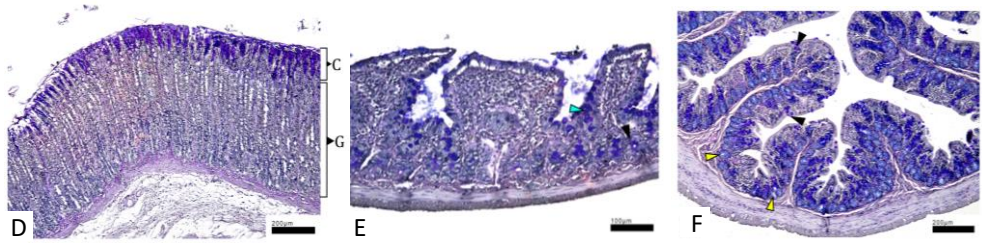


Fig. 4 Histochemical appearance (PAS - alcian blue coloration) of: D) gastic mucosa (group 3, subacute toxicity) - gastric pits (C) and fundic glands (G) with no structural modifications, normal functioning of cells secreting neutral mucins found on the surface epithelia of the stomach; E) small intestine mucosa (group 2, subacute toxicity) - normal functioning of Goblet cells secreting neutral mucins (blue arrowhead) and acid mucins (black arrowhead); F) colonic mucosai (lot 3, toxicitate subcronică) - (group 3, subchronic toxicity) - PAS positive cells (black arrowhead) and DCS cells (yellow arrowhead)

Consequently, we can say that the *I. helenium* extract passes through the entire digestive tract without causing structural changes in the tubular organs of the

digestive system, at least not structural changes detectable by optical microscopy, at doses of 300 mg/kg and 600 mg/kg. The histochemical examination (PAS - alcian blue coloration) of the gastric, small intestine and colonic mucosa revealed the lack of irritating or toxic action of the extract on the examined organs, the mucins' production not being influenced by the dose or duration of administration of the extract (Fig. 4).

The histological examination of the liver sections (TG coloration) revealed the absence of morphological changes detectable by optical microscopy, demonstrating that the substances contained in the extract do not possess hepatotoxic action. During the immunohistochemical analysis of the hepatic tissue (Fig. 5), the expression of anti-Caspase-3 biomarker presented variations between the tested groups, the percentage of cells in apoptosis being 0.64% for the group 1, 4.32% in group 2 and 6.55% in group 3 of subacute toxicity study. In the subchronic toxicity study, the percentage of liver cells in apoptosis was 6.55% in the group 1 , 0.66% in group 2 and 1.10% in group 3. Following the analysis of the obtained results, no direct correlation was observed between the extract dose administered and the percentage of hepatocytes in apoptosis.

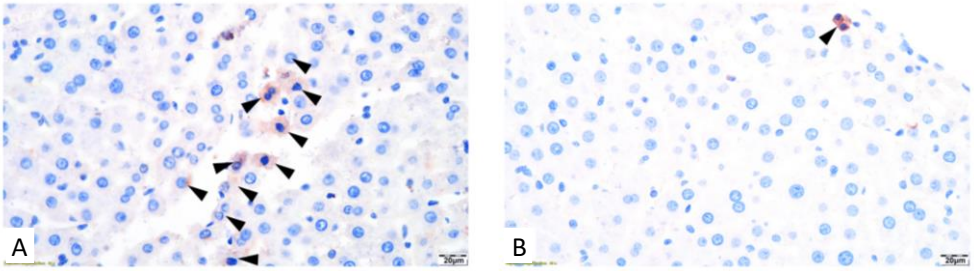


Fig. 5 Expression of anti-Caspase-3 biomarker at the level of liver (Wistar rats; Mayer hematoxylin counter-staining). A, B – group 2 subacute and subchronic study: intracytoplasmic expression of anti-Caspase-3 marker at the level of hepatocytes (arrowhead)

During the histological analysis of renal tissue (TG coloration) we observed the morphological aspect of renal corpuscles and proximal tubules, structural elements sensitive to systemic changes and presence of toxins in the body. The parietal layer of glomerular capsule and glomerular capillaries presented no microscopic modifications, the capsular space was transparent, and the proximal tubules did not contain exfoliated cells or debris in the lumen.

Hematologic and biochemical analysis of the blood. The insignificant oscillations of the investigated parameters indicate the absence of structural and functional changes or their inclusion within physiological limits, which did not exceed the adaptation capacity of the animal body.

RECOMMENDATIONS

Given the lack of data on the mechanisms of ovicidal and larvicidal action, toxic potential and therapeutic value of aqueous extracts of *I. helenium*, *A. absinthium*, *G. asclepiadea*, *T. vulgare* and *A. millefolium*, we recommend further *in vitro* and *in vivo*

studies, aimed to determine the bioactive metabolites responsible for their anthelmintic activity.

We believe that the complex protocol for *in vitro* and *in vivo* evaluation of the chemical composition, therapeutic and toxic potential of plant extracts, developed and applied by us, represents an appropriate testing model. Because of this we emphasize the importance of:

- Establishing the correlation between the chemical composition and the *in vitro* biological activity of plant extracts, in order to identify the bioactive compounds or classes of chemical compounds responsible for their therapeutic effect;
- Performing *in vitro* cytotoxicity studies prior to *in vivo* toxicity testing to determine the effect of extracts on target cells/tissue and anticipate the occurrence of adverse effects *in vivo*;
- The use of complex acute, subacute and subchronic toxicity study models, which include complex ante- and postmortem investigations in order to establish as accurately as possible the concentrations usable in the therapy with plant extracts.

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