UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

THE EXPERIMENTAL EVALUATION OF THE ADVERSE EFFECTS OF SOME DRUGS ADMINISTERED UNDER A MICROENCAPSULATED FORM

PhD thesis summary

SCIENTIFIC COORDINATOR
PROF. DR. Carmen Lăcrămioara ZAMFIR

PhD CANDIDATE
Ionuț DRAGOSTIN

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Keywords: *tuberculosis, hepatotoxicity, isoniazid, microparticles, biocompatibility*

The PhD thesis includes:

- 142 pages - of which 40 pages in The general considerations part;
- 54 Figures – of which 52 in The personal contribution part;
- 19 Tables – of which 11 in The personal contribution part;
- 338 bibliographical references.

In the present summary, the contents, the numbering of the selected figures and the list of abbreviations are kept in the same form as in the PhD thesis.

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ABBREVIATIONS USED

DNA	*Deoxyribonucleic acid*
ALAT	*Alanine aminotransferase*
APC	*Antigen presenting cell*
RNA	*Ribonucleic acid*
ASAT	*Aspartate aminotransferase*
ATP	*Adenosine triphosphate acid*
COPD	*Chronic obstructive pulmonary disease*
CLMW	*Chitosan low molecular weight*
CMC-Na	*Sodium carboxymethylcellulose*
MIC	*Minimum inhibitory concentration*
CMMW	*Chitosan medium molecular weight*
COX	*Cyclooxygenase*
LD 50	*Lethal dose 50*
DMSO	*Dimetyl sulfoxide*
DPPH	*1,1-diphenyl-picryl-hydrazyl*
DTG	*Differential thermogravimetric analysis*
FT-IR	*Fourier transform infrared spectroscopy*
GFR	*Glomerular filtration rate*
GST	*Glutathion S-transferase*
HGF	*Hepatocyte growth factor*
INH	*Isonicotinic acid hydrazide*
ACE inhibitor	*Angiotensin converting enzyme inhibitors*
Ig	*Immunoglobulins*
IL-6	*Interleukin-6*
BMI	*Body mass index*
Ly	*Lymphocytes*
MEM	*The minimum essential medium*
MHC	*Major histocompatibility complex*
MSR	*Membrane Swelling Ratio*
mtFAO	*Mitochondrial Fatty Acid Oxidation*
NAT	*N-acetyltransferase*
NGF	*Nerve growth factor*
OD	*Optical density*
WHO	*World Health Organization*
PAF	*Platelet aggregation factor*
PAS	*Para-aminosalicylic acid*
PEG	*Polyethylene glycol*
PG	*Prostaglandins*
NTCP	*National Tuberculosis Control Program*
PZA	*Pyrazinamide*
ADR	*Adverse drug reactions*
RIF	*Rifampicin*
ROS	*Reactive oxygen species*
TB	*Tuberculosis*
TG	*Thermogravimetric analysis*
TGF	*Transformed growth factor*
GOT	*Glutamic-oxalacetic transaminase*
GPT	*Glutamic pyruvic transaminase*
TMP-SMX	*Trimethoprim + sulphamethoxazole*
TNF-α	*Tumour necrosis factor*

INTRODUCTION

Predictable side effects (Type A) represent about 80% of the total adverse reactions and the unpredictable ones (Type B) affect more than 10% of the global population, while in the case of hospitalized patients the percentage increases to more than 20%. The category of predictable reactions include the toxic ones (which occur because of overdoses), adverse effects, side effects, drug interactions, while the unpredictable ones include: intolerance, idiosyncrasy, allergies and non-allergic hypersensitivity *(Thong B. Y-H., Tan T.C., 2011).* One of the most common adverse effects, in the case of chemical substance administration, of medicines used voluntarily or not, of over dosage, or even of drugs administered in therapeutic doses, is hepatocyte injury, which is why, in the drug industry, the experimental compounds are assessed before and during clinical trials, so that only compounds that prove to be safe for commercial use will be approved.

Tuberculosis, one of the most long-standing widespread diseases worldwide, represents an important cause of death globally. When treatment is appropriate, tuberculosis, caused by drug-sensitive strains, can be cured in most cases, while in the absence of treatment, it can become fatal. Nowadays, tuberculosis therapy encompasses a very broad therapeutic spectrum, the administration of which entails a number of important side effects such as toxic or hypersensitivity reactions. This is one of the reasons why the introduction of new active substances in the treatment of tuberculosis, appears to be an extremely important factor in defining the evolution of the current therapy.

In order to deepen and develop the above-mentioned facts, the present study aims at reducing the adverse reactions caused by drug toxicity at the tissue level, by using the microencapsulation method, with the help of different in vitro and in vivo assays. Through an original and interdisciplinary approach, the present study aims at pursuing the following research directions: *(i)* the encapsulation of isoniazid and of its new derivatives, using chitosan as encapsulation material, in view of using the obtained samples when performing in vitro and in vivo assays within this paper; *(ii)* determination of acute toxicity (LD 50) of isoniazid derivatives through in vivo assays; *(iii)* establishing the new isoniazid derivative microencapsulation effect on chronic toxicity at the tissue level; *(iv)* conducting in vitro studies on antimicrobial screening on strains of *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *(v)* determining the in vitro biocompatibility of both non-encapsulated and encapsulated forms by determining cell viability.

PERSONAL CONTRIBUTION

Chapter 5
MATERIALS AND METHODS

5.1. Biological evaluation and pharmaco-toxicological profile of isoniazid and its derivatives, administered as an oral suspension and in a microencapsulated form

New compounds with a hydrazonic structure (INH-a, INH-b, INH-c) were assessed from a biological and pharmaco-toxicological point of view in order to determine their antimicrobial potential, by using *Mycobacterium tuberculosis ATCC 25177* as a test strain, the acute toxicity by establishing the LD 50, as well as the chronic hepatotoxicity level, by using in vivo tests, administering them as an oral suspension, to Swiss white male mice. In addition, in vitro biocompatibility was determined by analysing the viability of the MTT cells.

5.1.1. <In vitro> evaluation of antimicrobial activity

5.1.1.1. The absolute concentrations method

The study was carried out in collaboration with the Clinical Hospital of Pneumophysiology, the Medical Tests Laboratory, Iași. This assay was performed for simple isoniazid (INH) and its three derivatives, with an isonicotinoyl hydrazone structure (INH-a, INH-b and INH-c), using *Mycobacterium tuberculosis ATCC 25177* as a microbial strain. The purpose of this determination is to verify the influence that the chemical modification has on the structure of the isoniazid, on the antimicrobial potential. Thus, dilutions of 1, 2 and 4 μg / ml in DMSO (dimethyl sulfoxide) were used: dilutions of 1 and 2 μg / ml for INH (known in the domain-specific literature as being the active ones for this compound - *Agalloco JP, Carleton FJ , 2007*), while for the INH -a, INH -b and INH -c derivatives, all three dilutions were tested. The growth medium used for this method was a solid, egg-based, Lowenstein-Jensen one.

5.1.1.2. Determination of Minimum Inhibitory Concentrations (MIC)

In addition to the absolute concentrations method, in order to assess the influence of the chemical changes made in the structure of isoniazid on the antimycobacterial activity, once the new synthesized compounds were obtained, the active minimum inhibitory concentrations (MIC) were determined with respect to the *Mycobacterium tuberculosis* strain, ATCC 25177. This parameter (MIC) is a very important factor in establishing the dosage and routes of administration in the case of infections caused by the Koch bacillus.

5.1.1.3. Determination of the Inhibition Zone Diameter

The antibacterial and antifungal activity of the new compounds was assessed in vitro, by using the agar disk-diffusion method, according to the CLSI (Clinical and Laboratory Standard Institute. Performance Standards for Antimicrobial Susceptibility Testing) specifications. 27th ed. CLSI supplement M100, 2017; Clinical and Laboratory Standard Institute. Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline. Second Edition, 2009).

5.1.2. Performing the toxicological screening. Determination of acute and chronic toxicity

The isoniazid derivatives (INH-a, INH-b and INH-c), obtained and described above, were assessed in vivo and compared to isoniazid (INH), from a toxicological point of view, determining the lethal dose 50. The study was performed in collaboration with the subject-matter Pharmacology, Faculty of Medicine, University of Medicine and Pharmacy "Grigore T. Popa", Iași.

The hosting, handling, administration of the compounds tested as an oral suspension and in a microencapsulated form, as well as the slaughter of the animals, were carried out according to the guides regarding the deontology and ethics of laboratory animals study (Law no. 206/27th of May 2004, EU / 2010/63 - CE86 / 609 / EEC) and after obtaining the approval of the "Grigore T. Popa" UMP Research Ethics Commission, Iași, issued on 17.04.2018.

Acute toxicity, namely the determination of the lethal dose 50, consists in administering compounds in geometric progression concentrations in order to calculate the dose that kills 50% of the mice. The toxicity of pharmaceutical substances varies in inverse proportion with the obtained lethal dose 50 values. Thus, the higher the value of LD 50, the lower the toxicity of the compounds.

Chronic toxicity. Based on the results obtained during the acute toxicity test, the chronic toxicity of the obtained compounds was monitored, by their administration in doses of 1/10 of the LD 50, by means of oral gavage.

5.1.3. Carrying out the histopathological study

In the case of acute toxicity evaluation, fragments of brain, heart, kidney, lungs and liver were examined. In order to assess the effect of administering the selected compounds as an oral suspension, on the chronic tissue toxicity, a histopathological study was performed on liver tissue fragments, to identify any morphological changes. For the experimental evaluation of the selected compounds microencapsulation effect on the chronic tissue toxicity, a histopathological study was carried out on liver tissue fragments, in order to identify any morphological changes produced by the chronic administration of the encapsulated substances. The first step was to include tissue fragments taken from paraffin. The resulting paraffin blocks were sectioned with the help of a microtome, 2-3 microns thick, the sections obtained being exposed on the slides, after which they were specifically coloured with H&E. In order to see if there are tissue alterations that highlight the impact of administering the substances as an oral suspension of 1% CMC-Na, the microscopic examination was performed with the help of a Nikon Eclipse 50i microscope.

5.1.4. The evaluation of in vitro sample biocompatibility using the MTT cell viability assay

The study was carried out in collaboration with the National Institute for Research and Development for Biological Sciences, Bucharest. For the analysis of the biocompatibility and the sample cell morphology examination, we used a stabilized line of NCTC mouse fibroblasts (929 clones), grown in T-25 tissue culture flasks at a density of

4x104 cells / ml in the Minimum Essential Environment (MEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Biochrom) and 1% antibiotics (penicillin, streptomycin and neomycin, purchased from Sigma-Aldrich). The evaluation of the cytotoxicity of the samples in a microencapsulated form was performed by exposing the cultured cells directly to the samples, followed by the MTT cell viability evaluation.

5.1.5. Evaluation of the biochemical parameters

The structural integrity of the liver is frequently assessed by determining the activity of serum aminotransferases (GPT and GOT) (*Amin A., Hamza AA, 2005*) so that, in order to investigate the extent of liver injury in the chronic administration of microencapsulated substances, serum levels of the following enzymes were determined: GPT, GOT and alkaline phosphatase. In addition, the total serum cholesterol and the serum albumin were also determined and the data obtained were uploaded and processed using the statistical functions of SPSS 18.0 at a significance level of 95%.

Chapter 6
RESULTS

6.1. Biological evaluation and pharmaco-toxicological profile of isoniazid and its derivatives, administered as an oral suspension

6.1.1. <In vitro> evaluation of antimicrobial activity

6.1.1.1. The absolute concentrations method

The results obtained for the evaluation of antimicrobial activity are shown in figure 6.1.

Fig. 6.1. Antimicrobial action of isoniazid and of its derivatives (INH-a, INH-b and INH-c)

The growth of the tubercle bacilli is very slow, the first colonies begin to appear on the solid medium after 10-15 days, becoming mature only after 3-4 weeks. On the Löwenstein-Jensen medium, used in this test, the *M. tuberculosis* colonies look like dry, prominent, yellowish colonies. From test tube no. 1 up to test tube no. 11 there was no growth, fact which led us to declare the strain sensitive to the dilutions tested (1, 2 and 4 μg / ml). On the other hand, the solvent used in preparing the dilutions of the tested

substances (test tube 23), after 28 days of incubation, allowed the growth of the *Mycobacterium tuberculosis ATCC 25177* bacterial strain.

6.1.1.2. Determination of the Minimum Inhibitory Concentrations (MIC)

Table 6.I. In vitro activities of INH and of its derivatives against *M. tuberculosis*

Compound	**MW (Molecular Weight)**	**MIC (µg/ mL)**	**MIC (µM)**
INH	137,14	0.12	0.87
INH-a	225,25	0.84	3.72
INH-b	270,25	4.166	15.41
INH-c	304,15	1.785	5.868

All compounds are active on the reference strain, *Mycobacterium tuberculosis ATCC 25177*, at different Minimum Inhibitory Concentrations, which means that the condensation reaction with the three benzaldehydes does not adversely affect the therapeutic effect of isoniazid. Therefore, the compounds can be considered biologically active and with potential use in the treatment of tuberculosis.

6.1.1.3. Determination of the Inhibition Zone Diameter

The chemical modifications made to the functional amino group of isoniazid, that is to say the blocking by condensation reactions with benzaldehydes, changed the biological profile of the obtained INH derivatives. The obtained results show, for isoniazid and its derivatives obtained by synthesis, an extended antimicrobial spectrum, unlimited for the *M. tuberculosis* strains, including Gram positive and fungal bacteria. Isoniazid and its synthesized derivatives exhibited no action against the Gram negative bacteria tested. The results also demonstrate that different structural modulations of isoniazid can favourably influence antimicrobial activity.

Table 6.II. Diameters of the microbial growth inhibition zones (mm) for Gram positive, Gram negative bacteria and fungi

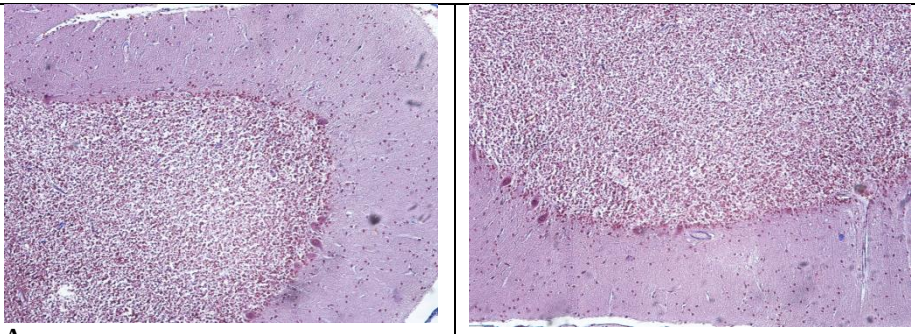
Sample	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. albicans* ATCC 14053
INH	13	0	0	15	16
INH-a	15	0	0	13	15
INH-b	15	0	0	12	13
INH-c	12	0	0	12	14
DMSO	0	0	0	0	0
Ciprofloxacin 5µg/disk	26	30	25	-	-
Fluconazole 25 µg/disk	-	-	-	28	29
Voriconazole 1 µg/disk	-	-	-	25	24

6.1.2. Performing the toxicological screening. Determination of acute toxicity

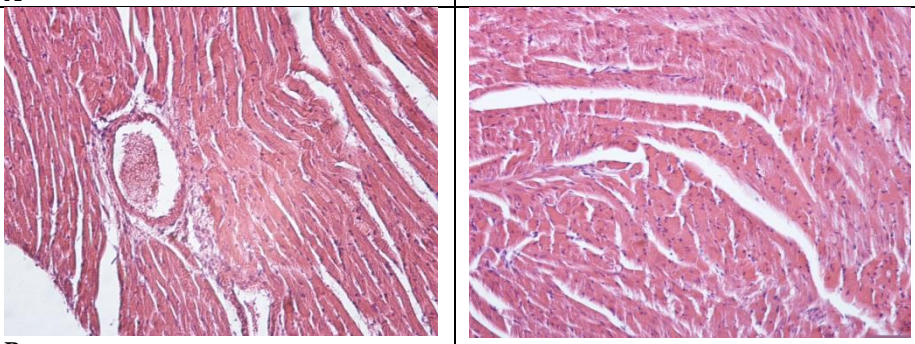
According to the obtained results, one may notice that the toxicity of the INH-a, INH-b and INH-c derivatives is lower, having values of LD50 in the range of 352.34-1778.8 mg / kg body, as compared to isoniazid, the initial compound (INH), whose LD50 is 175.2 mg / kg body weight. Thus, by the condensation of isoniazid with the three aromatic benzaldehydes, a significant reduction of its acute toxicity was obtained, the resulting compounds, i.e. INH-b and INH-c, being able to be classified as substances with moderate toxicity, while INH-a, together with the simple isoniazid (INH), may be included in the category of highly toxic substances.

Histopathological study (acute toxicity)

In the case of LD evaluation, fragments of brain, heart, kidney, lungs and liver were examined (Figure 6.5), taken from the laboratory animals used in the acute toxicity assay. In comparison with the Control group, the administration of isoniazid doses did not cause changes at the cerebral, cardiac and liver level. In contrast, at a pulmonary and kidney level, certain changes occurred. Thus, the pulmonary drawing is accentuated, with the destruction of the alveolar walls and spaces, vascular congestion and areas of inflammation. At the kidney level, tubular necrosis occurred, accompanied by inflammatory areas, which extend deep into the renal parenchyma, most often following the pathway of the kidney tubules.



A



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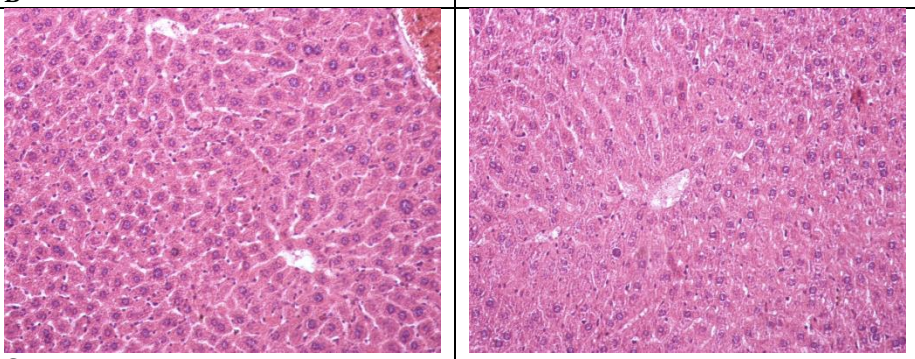

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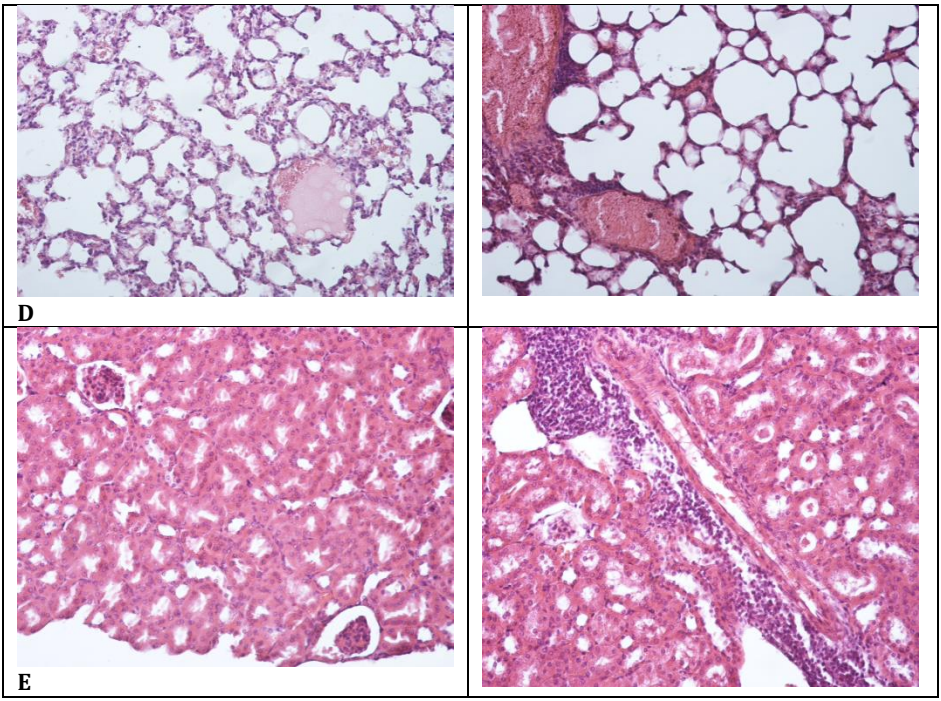

Fig. 6.5. Reactivity of the brain (A), cardiac (B), liver (C), pulmonary (D) and renal (E) tissue to lethal dose evaluation in the Control group / LD group. Col. HE, x20

6.1.3. Determination of chronic toxicity. Carrying out the histopathological study

In order to assess the impact of the analysed compounds administration, administered in a free, non-encapsulated form, on the induced liver injury, liver fragments were examined, taken from the first 6 groups studied and detailed in the Materials and methods section. The histopathological study of the liver fragments taken from each group allowed the evaluation of the changes induced by the chronic administration of isoniazid and of its derivatives, as an oral suspension.

The untreated Control group (group 11) revealed a normal liver morphology, with regular uni- or binucleate hepatocyte cords, radiating from the centrilobular vein, sinusoids of normal appearance and a normal configuration of the whole hepatic parenchyma (Fig.6.6, Fig. 6.7).

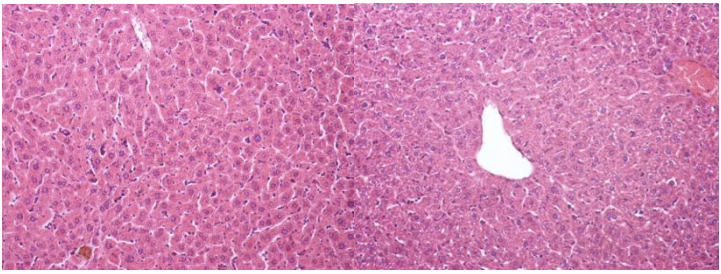

Fig. 6.6. Normal hepatic morphology **Fig. 6.7.**Centrilobular vein, radiating hepatocyte cords

Group 1 (that received INH) showed extended liver injury: the hepatic parenchyma has extensive areas of inflammation and cell necrosis (Fig. 6.8, Fig. 6.9), as well as well-defined areas of microvesicular steatosis, which proves the toxicity of isoniazid on the liver tissue.

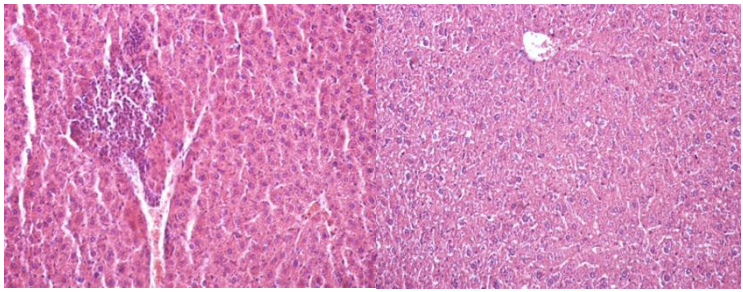

Fig. 6.8. Liver-areas of cell necrosis **Fig. 6.9.** Liver-Centrilobular vein

In this case, the liver section highlights the existence of microvesicular steatosis, a type of injury which is, nevertheless, considered to be reversible (*Andert A., et al., 2017*) after treatment discontinuation. Thus, hepatocytes have small, optically empty vesicles in the cytoplasm, with well-defined boundaries around the nucleus.

Group 2 (that received INH-a) also showed signs of liver tissue injury, revealed by frequent vascular congestion, diffuse inflammation and an onset of cell necrosis. Vascular congestion is caused by the accumulation of red blood cells in the lumen of the blood vessels.

Group 3 (that received INH-b) revealed the presence of perivascular lymphocyte inflammatory infiltrate as well as peri- and intrasinusoidal inflammation, capillary congestion, necrosis manifested by pyknotic nuclei and / or nuclear dots.

Group 4 (that received INH-c) showed the lowest signs of hepatic parenchyma alteration, as compared to the other similar groups: only slight vascular congestion. The normal morphology in this case is characterized by the presence of hepatocytes arranged in the form of radial cords, having uniform dimensions, with eosinophilic and / or granular

cytoplasm, focally with optically empty cytoplasmic vacuoles and one or more centrally located, round / oval nuclei, vascular congestion being reduced.

Group 5 (that received a simple solution of CMC-Na 1%) showed no changes in the liver cytoarchitecture. The normal morphology in this case is characterized by the presence of hepatocytes, with uniform dimensions, with eosinophilic and / or granular cytoplasm, focally with optically empty (fat) cytoplasmic vacuoles and one or more central, round / oval nuclei. Hepatocytes are arranged in the form of cords that converge towards the centrilobular vein, with intraluminal haematic infiltrate.

6.1.4. The evaluation of in vitro sample biocompatibility using the MTT cell viability assay

The evaluation of in vitro biocompatibility by contact with NCTC mouse fibroblast samples (929 clones), over a 24-hour period, indicates an increased cell viability, in the case of isoniazid derivatives, tested at the final concentration of 1.75 mg / mL. Thus, in the case of INH-b and INH-c samples, cell viability is higher (87.73%, 88.97% respectively) compared to that of the negative INH control (84.02%).

After 48 and 72 hours of testing, the analysed powders, especially INH-a and INH-b, induced an increasing cytotoxicity, with cell viability values ranging from 55.30% to 61.16% (at 48 hours) and 50.58% and 53.14% (at 72 hours), values similar to those of the reference INH compound (55.15% at 48 hours and 46.04% at 72 hours) (Table 6.IV); all these values situate the tested samples at the boundary between the light cytotoxic and moderately cytotoxic compounds. The most non-cytotoxic sample, at all three intervals (24, 48 and 72 hours), was INH-c, for which a cell viability of 88.97% was recorded (at 24 hours), and of 72.47%, respectively (at 72 hours). These values place the INH-c compound at the boundary between non-cytotoxic and slightly cytotoxic compounds. Of all the analysed samples, i.e. all the three isoniazid derivatives, the highest proliferation rate, similar to that of the untreated Control group, is presented by the INH-c compound. The aspect of cell morphology, when using this sample, confirms the viability result obtained by the MTT assay, thus suggesting a good biocompatibility with NCTC fibroblasts.

6.1.5. Evaluation of the biochemical parameters

6.1.5.1. Alanine aminotransferase (ALAT) / Glutamic-Pyruvic Transaminase (GPT)

The cellular integrity of the liver of the animals included in this study was evaluated by testing the activity of liver enzymes: GPT, GOT, alkaline phosphatase, total serum cholesterol and serum albumin.

In the case of substance administration in a non-encapsulated form, in a suspension of CMC-Na 1% (0.1 ml / kg body), after analysing the obtained results, the highest average values of GPT were found in the group of animals that received isoniazid (106.20 U / L- lot 1-HIN), significantly higher values compared to those recorded in the other studied groups, which received the corresponding derivatives: INH-a (91.09 U / L),

INH-b (86.75 U / L) and INH-c (84.87 U / L). The lowest mean GPT values were found in the untreated group (23.70 U / L-lot 11) and the CMC-Na group 1% (0.1 ml / kg body) (27.01 U / L-lot 5).

6.1.5.2. Aspartate aminotransferase (ASAT) / Glutamic-Oxaloacetic Transaminase (GOT)

Regarding the GOT values, in the case of the administration of the suspended substances of CMC-Na 1% (0.1 ml / kg body), after analysing the obtained results the highest average values were found in the group of animals which received isoniazid (166.58 U / L - lot 1-INH), higher values compared to those recorded in the other studied groups, which received the corresponding derivatives: INH-a (154.89 U / L), INH- b (152.18 U / L) and INH-c (151.21 U / L). The lowest mean TGO values were found in the untreated Control group (74.76 U / L - lot 11) and the CMC-Na group 1% (0.1 ml / kg body) (73.87 U / L - lot 5).

6.1.5.3. Alkaline phosphatase

The highest mean values of alkaline phosphatase were found in the INH group (189.74 U / L- lot 1), significantly higher values than those recorded in the CMC-Na groups (96.69 U / L; $p = 0.001$) and the untreated Control group (97.21 U / L; $p = 0.001$), where the lowest mean values of alkaline phosphatase were recorded.

6.1.5.4. Total cholesterol

The highest mean values of total serum cholesterol were found in the INH group (57.64 mg / dl-lot 1), higher than those recorded in the CMC-Na groups (51.24 mg / dl; $p = 0.001$) and the untreated Control group (51.66 mg / dl; $p = 0.001$), where the lowest average cholesterol values were recorded (Fig. 6.22). For the groups that received the other non-encapsulated isoniazid derivatives, the total serum cholesterol was between 54.07 and 54.84 mg / dl.

6.1.5.5. Serum albumin

The highest mean serum albumin values were found in the CMC-Na group (4.61 g / dl-lot 5) but also in the group of animals receiving INH (4.18 g / dl-lot 1). The lowest serum albumin values were recorded in the group of animals receiving INH-a (3.23 g / dl-lot 2), values close to those recorded in the animals from the untreated group (3.15 g / dl-lot 11).

6.2. Evaluation of the pharmaco-toxicological profile of isoniazid and its derivatives, administered in a microencapsulated form

6.2.1. Determination of chronic toxicity. Carrying out the histopathological study

The histopathological study of the liver fragments taken from each group allowed the evaluation of the changes induced by the chronic administration of isoniazid and of its derivatives, as an oral suspension (presented in subchapter 6.1), as compared to the administration of these compounds in an encapsulated form, presented in this subchapter.

Group 6 (that received INH microparticles) showed few signs of liver injury, manifested only by sinusoidal dilatation, with rare inflammatory reactions (intracapillary mononuclear inflammatory infiltrate) (Fig. 6.24, Fig. 6.25). In this case, the onset of cell necrosis is characterized by the presence of pyknotic nuclei and / or nuclear dots.

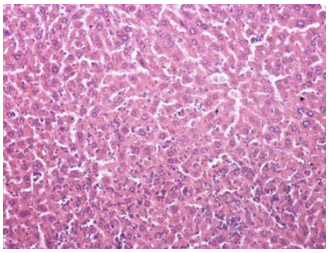

Fig. 6.24. Onset of necrosis

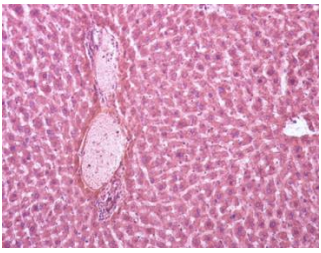

Fig.6.25. Small areas of perivascular inflammation

Group 7 (that received INH-a microparticles) also showed a pronounced dilatation of the sinusoidal capillary system, without necrosis, with rare inflammatory infiltrates (Fig. 6.26, Fig. 6.27). In addition, hepatocytes have a normal morphology, with regular sizes, the cytoplasm being eosinophilic and / or granular, focally with optically empty (fat) cytoplasmic vacuoles and one or more centrally located, round / oval nuclei. Also, there have been described areas of vascular congestion, characterized by frequent haematomas within the vascular lumen.

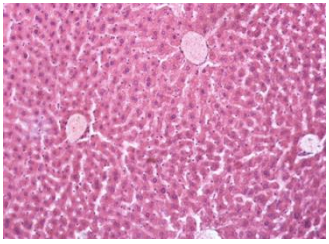

Fig. 6.26. Areas of vascular congestion

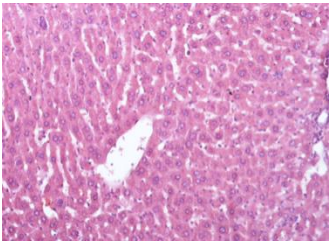

Fig. 6.27. Areas of reduced inflammation

Group 8 (that received INH-b microparticles) revealed areas of wide vascular congestion, characterized by abundant haematic infiltration inside the vascular lumen. In addition, one may notice the absence of cell necrosis areas, described in the case of the administration of the unencapsulated INH-b derivative. In conclusion, in the case of this group, the hepatic morphology is normal, presenting rare slightly dilated sinusoids.

For group 9 (that received INH-C microparticles) - part of the hepatocytes has a fine-vacuolar cytoplasm. Group 10, that received empty chitosan microparticles, the centrilobular vein and the hepatocytes have a normal morphology.

The untreated Control group 11 revealed a normal hepatic morphology, with cords of hepatocytes radiating from the centrilobular vein and a normal configuration of the hepatic parenchyma (Fig. 6.32).

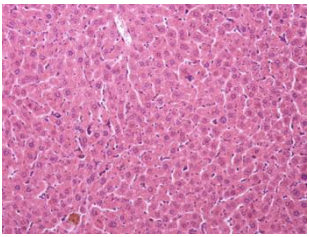

Fig. 6.32. Normal hepatic morphology

6.2.2. The evaluation of in vitro sample biocompatibility using the MTT cell viability assay

In the biocompatibility evaluation assay, the corresponding microparticles (CS-INH and CS-INH-a, b and c derivatives), displayed good biocompatibility for micro-encapsulated INH-b and INH-c derivatives, at all three intervals, having values of cell viability between 80.22 - 106.54%. The values recorded for the encapsulated derivatives were close to those of the empty chitosan (CS) microparticles, 90.92-98.32%, used as a negative control (table 6.V).

Comparing these results to those obtained in the case of the non-encapsulated derivatives, an improvement in cytotoxicity can be observed when using chitosan encapsulation, fact highlighted by higher values of the cell viability percentage. The derivative with the highest cellular viability at 72 hours, INH-c, showed, when used in an encapsulated form, a percentage of 94.14%, while in the non-encapsulated form the percentage was 72.47%.

Table 6.V. Cell viability (%) for isoniazid derivatives and the corresponding microparticles at 24 h, 48 h and 72 h

	Cell viability (%)		
Tested samples	**24 h**	**48 h**	**72 h**
Untreated Control group	100	100	100
H_2O_2 0.03% - positive control	11.99	5.02	2.90
CS (negative control)	98.23	97.10	90.92
CS-INH (reference compound)	100,27	98,37	95,53
CS-INH-a	83.92	71.08	63.99
CS-INH-b	106.54	99.09	89.64
CS-INH-c	94.96	80.22	94.14

(where: 80-100% is for non-cytotoxic compounds, 50-80% for mild cytotoxic compounds, 30-50% for moderate cytotoxicity and <30% for severely cytotoxic compounds) (ISO 10993-5, Geneva 2003).

6.2.3. Evaluation of the biochemical parameters

Liver cell integrity of the animals included in this study was evaluated by testing the activity of liver enzymes: GPT, GOT, alkaline phosphatase, total serum cholesterol, serum albumin.

6.2.3.1. Alanine aminotransferase (ALAT)/ Glutamic-Pyruvic Transaminase (GPT)

In the case of the administration of the substances in a microencapsulated form, a significant reduction of these values may be noticed, starting from the encapsulated isoniazid (group 6 - chitosan INH: 85.75 U / L) and up to the three encapsulated derivatives: chitosan INH-a (group 7 with 78.46 U / L), chitosan INH-b (group 8 with 76.20 U / L) and the lowest value recorded in the group that received chitosan INH-c (group 9 with 75.15 U / IT).

The lowest values of the GPT cytolysis indicator were found in the Control group that received empty chitosan microparticles (22.97 U / L-lot 10) as well as in the untreated Control group (23.70 U / L-lot 11).

6.2.3.2. Aspartate aminotransferase (ASAT)/ Glutamic-Oxaloacetic Transaminase (GOT)

In the case of the administration of the substances in a micro-encapsulated form, a slight decrease in these values is observed, starting from the encapsulated isoniazid (group 6 - chitosan INH: 159.58 U / L) and up to the three encapsulated derivatives: chitosan INH-a (group 7 with 147.13 U / L), chitosan INH-b (group 8 with 145.06 U / L) and chitosan INH-c (group 9 with 145.18 U / L). The lowest values of the GOT indicator were found in the Control group that received empty chitosan microparticles (76.28 U / L) as well as in the untreated Control group (74.76 U / L - lot 11).

6.2.3.3. Alkaline phosphatase

The highest mean values of alkaline phosphatase were found in the encapsulated INH group (182.06 U / L- lot 6), significantly higher than those recorded in the CMC-Na groups (96.69 U / L; p = 0.001), chitosan Control group (91.08 U / L; p = 0.001) and

untreated Control group (97.21 U / L; p = 0.001) that had the lowest mean values of alkaline phosphatase. In the case of isoniazid derivatives administered in an encapsulated form (groups 7-9), there is a slight decrease in alkaline phosphatase, with values ranging between 175.44 and 179.56 U / L.

6.2.3.4. Total cholesterol

The highest mean value of total serum cholesterol was found in the encapsulated INH group (56.98 mg / dl- lot 6), higher values than those recorded in the chitosan Control group (51.12 mg / dl; p = 0.001) and the untreated Control group (51.66 mg / dl; p = 0.001) that had the lowest average cholesterol values (fig. 6.38). In the groups that received encapsulated INH-a, INH-b and INH-c isoniazid derivatives, the total serum cholesterol ranged from 53.11 to 54.08 mg / dl.

6.2.3.5. Serum a*lbumin*

The lowest mean values of serum albumin were found in the untreated group (3.15 g / dl-lot 11) but also in the groups of animals that received isoniazid derivatives in an encapsulated form, groups 6-9, with values ranging between 3.36 and 3.66 g / dl. The highest value of serum albumin was recorded by the group of animals that received empty chitosan microparticles, i.e. group 10 (4.28 g / dl).

6.2.4. Comparative study of the biochemical parameters on all 11 groups of animals (for non-encapsulated and encapsulated substances)

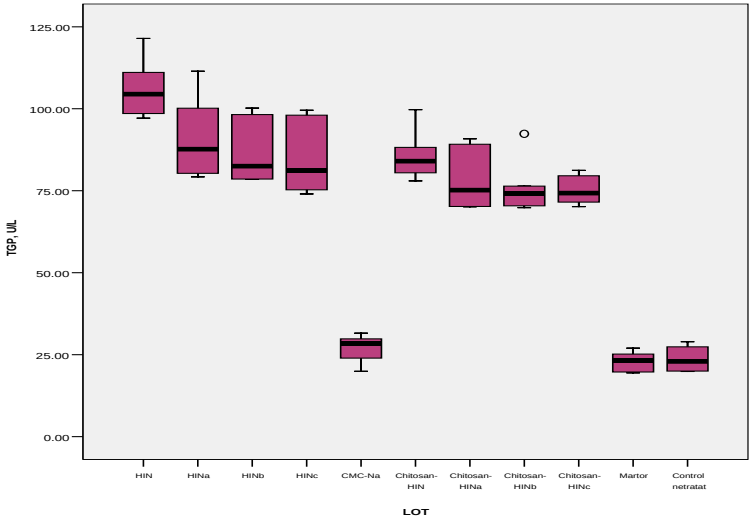

Fig. 6.40. Comparative GPT mean values for all study groups

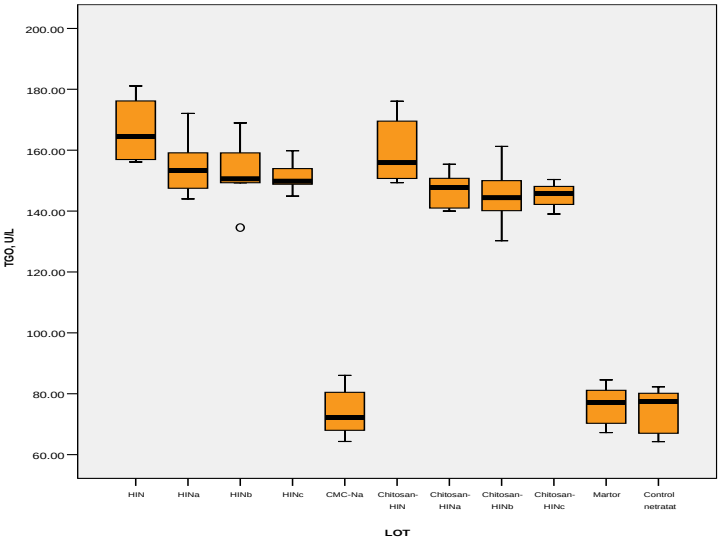

Fig.6.41. Comparative GOT mean values for all study groups

The mean value close to the group mean value suggests that the GPT value series was homogeneous, that is to say significance tests can be applied for the continuous variables. By comparing the GPT values of all the studied groups (Figure 6.40), one may notice a slight decrease in these values, in the case of the groups that received encapsulated substances, as compared to the groups that received the substances in an oral suspension.

The mean value close to the group mean value suggests that the GOT value series was homogeneous. As in the case of the GPT value determination, in the case of the GOT enzyme, too, after comparing all the values, one may notice a slight decrease in the values corresponding to the microcapsules containing the active substances (figure 6.41).

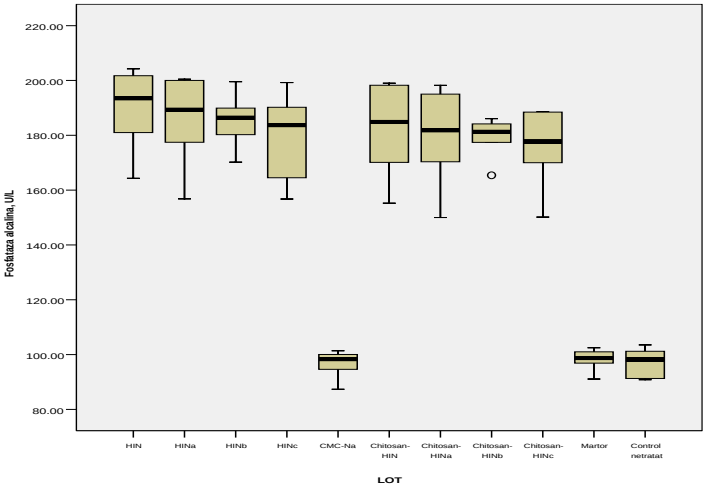

Fig. 6.42. Comparative alkaline phosphatase (ALP) mean values for all study groups

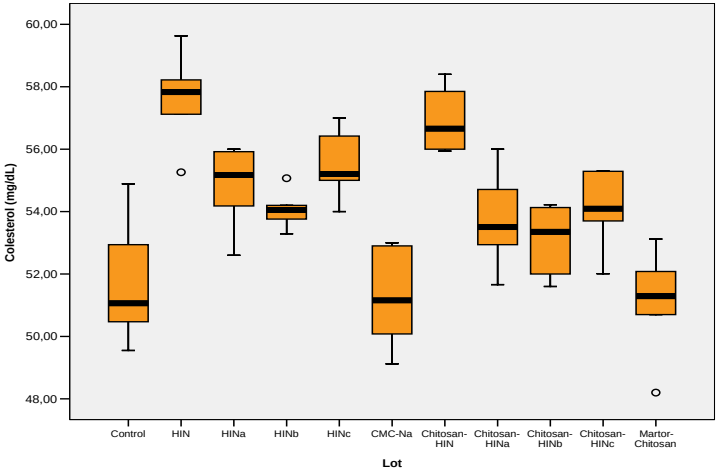

Fig.6.43. Comparative cholesterol mean values for all study groups

The closer the mean value is to the group mean value, the more homogeneous the range of values for ALP is. In this series of determinations, too, one may notice a slight decrease in the alkaline phosphatase, reaching a minimum of 175.44 U / L in the case of INH-c derivative encapsulation (figure 6.42).

The mean value close to the group mean value suggests that the total serum cholesterol value range was homogeneous, so significance tests can be applied for continuous variables. After comparing the cholesterol values of all studied groups (figure

6.43), a significant decrease may be noticed in the groups that received encapsulated substances as compared to the groups that received the substances in an oral suspension.

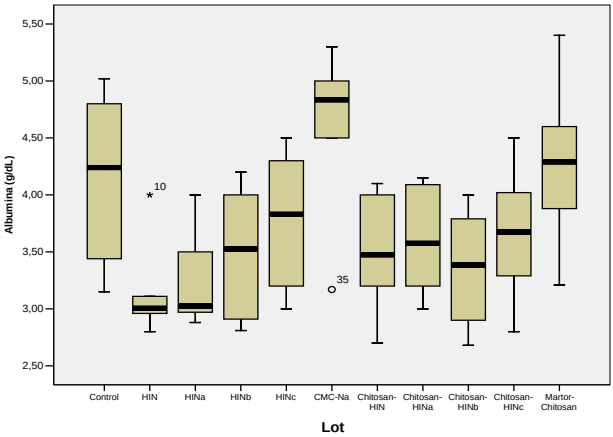

Fig.6.44. Comparative serum albumin mean values for all study groups

The mean value close to the mean value of the group suggests that the serum albumin value series was homogeneous, so significance tests can be applied for continuous variables. After comparing the serum albumin values of all studied groups (Figure 6.44), a significant increase in these values may be noticed in the groups that received encapsulated substances, as compared to the groups that received the substances in an oral suspension. This increase is all the more important as one sign of liver injury is hypoalbuminemia.

6.2.5. Correlation of liver markers in laboratory animals treated with non-encapsulated isoniazid and derivatives

In animals treated with INH and non-encapsulated derivatives, GPT recorded:

- direct, high intensity correlations with GOT ($r = +0.903$; $p = 0.001$), alkaline phosphatase ($r = +0.893$; $p = 0.001$) and cholesterol ($r = +0.812$; $p = 0.001$);
- indirect correlation, moderate in intensity, with albumin ($r = -0.660$; $p = 0.001$).

In animals treated with INH and non-encapsulated derivatives, GOT recorded:

- direct, high intensity correlations with alkaline phosphatase ($r = +0.913$; $p = 0.001$) and cholesterol ($r = +0.758$; $p = 0.001$);
- indirect correlation, moderate in intensity, with albumin ($r = -0.668$; $p = 0.001$).

In animals treated with INH and non-encapsulated derivatives, alkaline phosphatase was significantly correlated with:

- direct correlation, moderately-high in intensity, with cholesterol ($r = +0.708$; $p = 0.001$);
-indirect correlation, moderate in intensity, with albumin ($r = -0.695$; $p = 0.001$).

In animals treated with INH and derivatives, the correlation between cholesterol and albumin was indirect, moderate in intensity, statistically significant (r = -0.439; p = 0.015).

6.2.6. Correlation of liver markers in laboratory animals treated with chitosan-encapsulated isoniazid and derivatives

In laboratory animals treated with chitosan-encapsulated substances, GPT was significantly correlated with:

-GOT (r = +0.920; p = 0.001) and alkaline phosphatase (r = +0.924; p = 0.001), high intensity direct correlations, and cholesterol (r = + 0.665; p = 0.001), a moderate in intensity correlation;

- indirect correlation, a moderate in intensity correlation with albumin (r = -0.474; p = 0.008).

In laboratory animals treated with chitosan microparticles, GOT recorded:

- direct correlations, of high intensity, with alkaline phosphatase (r = +0.896; p = 0.001) and moderate with cholesterol (r = +0.671; p = 0.001);

- indirect correlation, moderate in intensity, with albumin (r = -0.464; p = 0.01).

In laboratory animals, treated with chitosan-microencapsulated substances, alkaline phosphatase was significantly correlated with:

-cholesterol, a direct, moderate in intensity correlation (r = +0.591; p = 0.001);

-albumin, an indirect, moderate in intensity correlation (r = -0.379; p = 0.039).

In chitosan-treated mice, the correlation between cholesterol and albumin was indirect, reduced in intensity (r = -0.221; p = 0.240), but the result could not be extrapolated to the general population.

Chapter 7
DISCUSSIONS

In the case of the administration of chemical substances, represented by medicines administered voluntarily or not, in overdose, or even when administered in therapeutic doses, the process of hepatocyte injury occurs. Also, hepatocytes can be affected by chemical substances, which can be used in laboratories or industrially, as well as by herbal remedies. Hepatotoxicity, as an adverse effect of drugs, is a common reaction, identified not only during the development of new molecular entities, with therapeutic effect, but also after the approval of the use of drugs, on a large scale, for different conditions. In preclinical toxicology studies, hepatotoxicity is a major obstacle to the development of new drugs, the liver being the most exposed organ to the adverse reactions induced by their use. In addition, compared to other tissues, the liver is the most exposed to high concentrations of a drug, when administered orally.

In the case of isoniazid, studied in this paper, after its biotransformation in the liver, the production of nitrogen-centred free radicals takes place, which generates reactive oxygen species that act as stimulants of lipid peroxidation, ultimately causing cell death and liver necrosis. Also, most hepatotoxic chemicals affect the liver mainly by

inducing lipid peroxidation, either directly or indirectly. In laboratory animals, peroxy radicals mediate lipid peroxidation, thus destroying cell membrane integrity, leading to liver injury, atherosclerosis and renal injury (*Singh C., et al., 2014*).

Improving the pharmaco-toxicological profile of isoniazid by introducing chemical modifications into its main structure in order to increase the biological response to *Mycobacterium tuberculosis*, to reduce liver toxicity or to avoid resistance phenomena, continues to be an increasingly intriguing scientific challenge.

As far as the chemical modifications are concerned, the aim was to convert hydrophilic isoniazid into a series of lipophilic derivatives, this being achievable by increasing the molecular weight of the resulting compounds, together with the addition of some hydrophobic hydrocarbons to the free amino group of the simple compound. As a result, increasing the molecular mass will increase the lipophilicity / hydrophobicity of the compound. This property could have a positive influence on the antimicrobial activity of the resulting compounds, but there are also studies that have shown that there may be other factors, besides the properties of hydrophobia, that influence the absorption and distribution of a tuberculostatic drug within the *M. tuberculosis* strain. Among the factors mentioned, one may list the structural properties of the compounds, the complex micro-environment inside the cell, as well as the cell wall permeability differences of different strains (*Parumasivam T., et al., 2013*). Therefore, the structure of the cell wall and the penetration capacity of isoniazid through the lipid barrier provide the opportunity for development strategies of new, less toxic and more efficient tuberculostatic drugs.

In our study, although from the point of view of the antimicrobial activity, it was found that the activity of isoniazid derivatives is slightly lower than that of the simple isoniazid, because of higher values of the minimum inhibitory concentration, they are within the limits imposed by the Global Alliance for New Antituberculosis Drug Development, namely 6.25 μg / ml, the upper limit for the evaluation of antituberculosis activity of the new therapeutic compounds (*Rodrigues MO, et al., 2013*).

In vitro biocompatibility showed a decrease in the cytotoxicity of isoniazid derivatives by encapsulation in chitosan, with up to 50% higher cell viability rates, as opposed to the non-encapsulated forms. In addition, the average cell viability, promoted by these derivatives (INH-a, INH-b and INH-c), was higher than that of isoniazid.

The condensation of isoniazid with the three aromatic benzaldehydes led to a significant decrease in acute toxicity, by recording up to ten times higher lethal doses. Thus, LD 50 increased significantly, from 175.2 mg / kg body (in the case of INH) to 1778.8 mg / kg body (in the case of the INH-b compound) and, therefore, based on the results obtained, it can be stated that the studied derivatives belong to the group of low toxicity substances. According to some studies, the high reactivity of the amino terminal group in the isoniazid structure appears to be responsible for the hepatotoxicity processes (*Castelo-Branco F.D., et al., 2018*). For this reason, the chemical modifications made to this functional group, that is to say the benzaldehyde condensation blocking reactions, have greatly changed the hepatotoxicity profile of the obtained INH derivatives.

In our case, in the acute toxicity assay, with the help of the histopathological analysis, it was found that the lethal doses of the simple isoniazid produced tubular

necrosis accompanied by inflammatory areas at the kidney level, which led to acute kidney failure, followed by the death of the animals. The presence of tubular lesions indicates that acute tubular necrosis, responsible for renal ischemia, is the cause of acute kidney failure. The encapsulation of the isoniazid derivatives analysed from a biological and pharmaco-toxicological point of view, using chitosan as a microencapsulation polymer, had beneficial effects on their chronic toxicity, significantly reducing its level. In the histopathological observations of the liver sections, especially the animals that received isoniazid, during the 30 days, presented hepatocellular injury, manifested by necrosis and microvesicular steatosis.

In the present study, the degree of liver injury, in the case of non-encapsulated compounds, was highlighted, on the one hand, by the significant increase, of approximately four times, in liver enzymes and, on the other hand, by the histopathological changes occurring in the liver, which were mentioned above. In our case, the use of isoniazid and of its corresponding derivatives, administered in a non-encapsulated form, increased the level of total cholesterol, while the use of chitosan in the encapsulated administration of these compounds, led to a decrease in these values, by means of the hypocholesterolemic effect, quoted in the domain-specific literature (*Naveed M., et al., 2019*).

In addition, liver injury is also proven by a decrease in albumin values, especially in the groups of animals that received non-encapsulated substances, so that the lowest values were recorded for animals that received INH administered as an oral suspension (non-encapsulated). In the case of the INH-a, INH-b and INH-c derivatives, there is a slight increase in albumin values, while the encapsulation of these compounds causes a significant increase in values, reaching values close to that recorded in the untreated Control group, fact which suggests the positive effect of chitosan on serum albumin, as well as the chemical changes produced by the simple isoniazid.

Chapter 8
CONCLUSIONS

The obtained results showed that the antioxidant action of chitosan can significantly reduce liver injury induced by drugs, by neutralizing the free radicals, by inhibiting lipid peroxidation, thanks to its electropositive charge, acting as a cation, as a result of which it can be fixed to the negatively charged cell membranes, causing some biological changes, by neutralizing the loss of the protective potential and the oxidative stress. In this context, the use of chitosan microencapsulation of different hepatotoxic drugs represents an important strategy in reducing liver injury and, therefore, reducing the risk of fulminant hepatic failure.

In conclusion, the obtained results have potential application as antimicrobial agents in the treatment of tuberculosis, where both a favourable antimicrobial action and a lower incidence of adverse reactions are required, especially in the context of the high toxicity of current tuberculostatic medication.

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