New isoniazid derivatives with improved pharmaco-toxicological profile: Obtaining, characterization and biological evaluation

Ionut Dragostin, Oana M. Dragostin, Sangram Keshari Samal, Saumya Dash, Rodica Tatia, Maria Dragan, Luminița Confederat, Cristina M. Ghiciuc, Daniela Diculencu, Cătălina E. Lupușoru, Carmen L. Zamfir

PII:	S0928-0987(19)30237-4
DOI:	https://doi.org/10.1016/j.ejps.2019.104974
Article Number:	104974
Reference:	PHASCI 104974
To appear in:	*European Journal of Pharmaceutical Sciences*
Received date:	28 December 2018
Revised date:	10 June 2019
Accepted date:	25 June 2019

Please cite this article as: I. Dragostin, O.M. Dragostin, S.K. Samal, et al., New isoniazid derivatives with improved pharmaco-toxicological profile: Obtaining, characterization and biological evaluation, European Journal of Pharmaceutical Sciences, https://doi.org/10.1016/j.ejps.2019.104974

NEW ISONIAZID DERIVATIVES WITH IMPROVED PHARMACO-TOXICOLOGICAL PROFILE: OBTAINING, CHARACTERIZATION AND BIOLOGICAL EVALUATION

Ionut Dragostin[#a], *Oana M. Dragostin**[#b], *Sangram Keshari Samal*[#c,i], *Saumya Dash*[c], *Rodica Tatia*[d], *Maria Dragan*[e], *Luminița Confederat*[f], *Cristina M. Ghiciuc*[g], *Daniela Diculencu*[h], *Cătălina E. Lupușoru*[g], *Carmen L. Zamfir*[a]

[a]University of Medicine and Pharmacy Grigore T. Popa, Faculty of Medicine, Department of Histology, 16 Universitatii Str., 700115, Iasi, Romania; ionut.dragostin@yahoo.com; zamfircia@yahoo.com;

[b]Dunarea de Jos University of Galati, Faculty of Medicine and Pharmacy, Research Centre in the Medical-Pharmaceutical Field, 47 Domneasca Str., Galati, Romania; oana.dragostin@ugal.ro

[c]Laboratory of Biomaterials and regenerative Medicine for Advanced Therapies, Materials Research Centre, Indian Institute of Science Bangalore- 560 012, Karnataka, India; sksamalrec@gmail.com; saumya.bulbul112@gmail.com;

[d]Romanian National Institute of Research and Development for Biological Sciences, 296 Splaiul Independentei, 060031 Bucharest, Romania; rodica.tatia@gmail.com;

[e]University of Medicine and Pharmacy Grigore T. Popa, Faculty of Pharmacy,16 Universitatii Str., 700115, Iasi, Romania; mwolszleger@yahoo.com;

[f]University of Medicine and Pharmacy Grigore T. Popa, Faculty of Medicine, Department of Microbiology, 16 Universitatii Str., 700115, Iasi, Romania; luminitza_kya28@yahoo.com;

[g]University of Medicine and Pharmacy Grigore T. Popa, Faculty of Medicine, Department of Pharmacology, 16 Universitatii Str., 700115, Iasi, Romania; c_ghiciuc@yahoo.com; celupusoru@yahoo.com;

[h]Clinical Pneumophysiology Hospital, Medical Analysis Laboratory, Iasi, Romania; ddiculencu@gmail.com

[i]Laboratory of Biomaterials and regenerative Medicine for Advanced Therapies, Indian Council of Medical Research-Regional Medical Research Centre, Bhubaneswar-751 023, Odisha, India.

Abstract: Tuberculostatic drugs are the most common drug groups with global hepatotoxicity. Awareness of potentially severe hepatotoxic reactions is vital, as hepatic impairment can be a devastating and often fatal condition. The treatment problems that may arise, within this class of medicines, are mainly of two types: adverse reactions (collateral, toxic or hypersensitive reactions) and the initial or acquired resistance of *Mycobacterium tuberculosis* to one or more antituberculosis drugs. Prevention of adverse reactions, increase treatment adherence and success rates, providing better control of tuberculosis (TB). In this regard, obtaining new drugs with low toxicity and high tuberculostatic potential is essential.

Thus, in this work, we have designed or synthesized new derivatives of isoniazid (**INH**), such as new Isonicotinoylhydrazone (**INH-a, INH-b** and **INH-c**). These derivatives demonstrated good biocompatibility, antimicrobial property similar to that of parent isoniazid and last but not least, a significantly improved Pharmacotoxicological profile compared to that of isoniazid.

Keywords: isoniazid, isonicotinoylhydrazone, tuberculosis, biocompatibility, antimicrobial, hepatotoxicity.

**Corresponding authors*: oana.dragostin@ugal.ro

[#] These authors have contributed equally.

1. INTRODUCTION

Tuberculosis (TB) is the deadliest infectious disease causes of 1.6 million deaths and around 10 million new TB cases registerd worldwide in 2017 alone (Global tuberculosis report 2018). TB is considered as the major challenge to the public health authorities in the countries where the infection and diagnosis can not be regulated appropiately. When the treatment is appropriate, tuberculosis caused by drug-sensitive strains is cured in most of the cases, and if the disease is not treated, it become fatal in up to 65% of cases, within 5 years of evolution (Loscalzo and Harrison, 2017). The risk of contamination and spread of *Mycobacterium tuberculosis* is high, due to its size, which leads to good aerosolization and to overcoming the respiratory barrier, entering into the lungs. According to growth conditions and age of the culture, bacilli may vary in size so that their dimensions have been reported to be 1-10 μm in length and 0.2 -0.6 μm width (Talip et al. 2013). Therefore, the aerogenic route is the most common way of transmitting tuberculosis infection from individual to individual (90%) (Yates et al. 2016). Antituberculosis therapy is being carried out today, by combining medication, this process being a standard in medical practice, aiming at the eradication of some resistant *Mycobacterium tuberculosis* species (Hoagland et al. 2016; Hu et al. 2017).

The therapeutic treatment of TB involves the adminstration of tuberculostatic drugs. However, these tuberculostatic drugs are the most common drug groups with global hepatotoxicity and the incidence of hepatotoxicity induced by them, varies greatly, depending on the drug regimens involved, the threshold used to define hepatotoxicity and reporting practices. In general, hepatotoxicity was reported in 5-28% of people treated with anti-TB medication (Vidyasagar and Guruprasad, 2012).

In this context, our attention stopped on isoniazid (INH) or Isonicotinyl hydrazine (arylhydrazine), one of the most widely used tuberculostatic drugs, which exhibits rapid oral absorption, reaching peak plasma concentrations within 1-2 hours of administration, with primary metabolism at the liver, via the N-acetyltransferase (NAT) enzyme system. Therefor, it is essential to take high doses of toxic INH, this process being directed towards an autoimmune reaction that affects the liver, causing high hepatotoxicity in the treatment with isoniazid (Susmita et al. 2016). For this major tuberculostatic drug, the most common side effect is the increase in the level of liver enzymes in the blood, which can lead to hepatitis. Hepatic toxicity caused by INH may occur as cellular necrosis, steatosis or both (Hossein et al. 2017).

The novelty of this work consists of developing a preclinical solution to reduce the risk of hepatotoxicity following tuberculostatic therapy with isoniazid, while maintaining its high therapeutic potential. Such a study conducted in a multidisciplinary context will contribute at the same time to the improvement of the obtained results as well as for increasing the impact on the scientific community. In this regard, three types of aromatic aldehydes were introduced: benzaldehyde, 2-Nitrobenzaldehyde, and Bromobenzaldehyde, with the objective of obtaining new INH condensation products, to minimize the acute and chronic tissue toxicity and improve the tuberculostatic potential. Regarding the development of new tuberculostatic agents, the obtaining of Isonicotinyl hyrazones has attracted the attention of researchers over time due to improved liposolubility (Sriram et al. 2005), their well established activities and clinical applications (Ahmad et al. 2005), while various studies have shown the effect of substances with different structures, on the *Mycobacterium tuberculosis* strain (Carroll et al. 2013; Iona et al. 2007; Carroll and Parish, 2015; Piccaro et al. 2013; Manetti et al. 2000; Da Silva Lourenço et al. 2008; Abdel-Aziz and Abdel-Rahman 2010; Maccari et al. 2005; Silva et al. 2006; Carvalho et al. 2008; Sriram et al. 2005; Navarrete-Vazquez et al. 2007; Bartzatt et al. 2012). In additon, physico-chemical characterization as well as *in vitro* (antimicrobial activity, biocompatibility) and *in vivo* biological evaluation (acute and chronic toxicity with histopathological study), complete the present work to highlight the impact of isoniazid chemical modification on the pharmacotoxicological profile improvement.

2. MATERIALS AND METHODS

2.1.General procedure for obtaining new hydrazones of INH

The condensation reaction between isoniazid and the three types of Aromatic Benzaldehydes Benzaldehyde (a), 2-Nitro-benzaldehyde (b) and 4-Bromo-benzaldehyde (c)) was carried out in a molar ratio of 1: 1. Thus, Isoniazid (0.0145 moles) is dissolved by heating in 50 ml absolute ethyl alcohol and over the solution thus obtained, 0.0145 moles of the corresponding aromatic benzaldehyde, is added. Over the resulting mixture, glacial acetic acid (2 mL) was added and then kept in a water bath under reflux for 8 hours. The resulting precipitate at the end of the reaction time was separated by filtration, washed with distilled water and dried at room temperature. The general synthesis scheme (**Figure 1**) has been developed by adopting some methods of condensation of similarly-structured compounds (Vosátka et al. 2018; Hu et al. 2017).

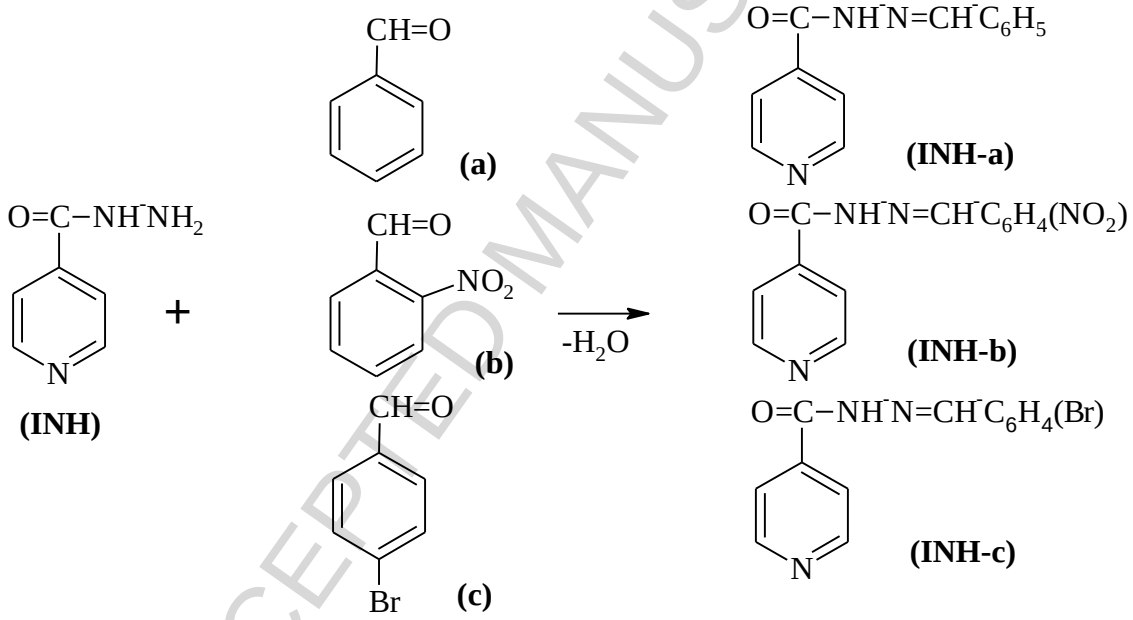


Figure 1. The general scheme for obtaining the three isonicotinoylhydrazones (INH-a, INH-b and INH-c).

2.2.Physico-chemical and spectral characterization of new INH derivatives

The condensation products of INH have been physically and chemically characterized, determining melting temperature, reaction yield, molecular formula and relative mass. Chemical structure confirmation, of the synthesized compounds was performed using *spectral methods:* Infrared Spectroscopy and Nuclear Magnetic Resonance (^{1}H-NMR).

2.2.1. <u>Attenuated Total Reflectance FTIR (ATR-FTIR)</u>

The chemical structural analysis of INH and derivatives of INH were evaluated by ATR-FTIR analysis. The experiment was performed with a JASCO FTIR-6200 (Tokyo, Japan) equipped with a MIRacle attenuated total reflection (ATR) Crystal Ge (IR penetration, 0.66 mm) cell in reflection mode, and absorbance spectra were collected at 32 scans coded with 4 cm^{-1} resolution in the region 4000–450 cm^{-1}. (Samal et al. 2014; Samal et al. 2014; Samal et al. 2015)

2.2.2. ^{1}H NMR spectroscopy analysis

^{1}H NMR spectra of INH and its derivatives were recorded with a JEOL NMR spectrometer 500 MHz using 5 mm LN2 cooled-probe. The samples were dissolved in DMSO-D6 (99.9% D) and ^{1}H NMR spectra were recorded at 298 K with 32 scans. The chemical shifts were expressed in Parts Per Million (PPM).

2.3.X-ray diffraction (XRD) study

The XRD of newly synthesized hydrazones were compared with that of pure isoniazid. The diffraction patterns were collected using an X-ray diffractometer (XRD; X'PertPro PANanalytical) working in the Bragg–Brentano configuration and equipped with a Cu Kα radiation (λ=1.5417 A$^{\circ}$), a diffracted beam monochromator and a scintillation detector. The samples were scanned at room temperature over a 2θ range of 5–60° C with a step size of 0.028 and a time per step of 2s. (Samal et al. 2014; Samal et al. 2014)

2.4.Thermogravimetric Analysis (TGA)

TGA experiments were performed with a TA Instruments, Q500 thermo-balance with Thermogravimetric Analyzer software (Universal Analysis 2000). Sample weights were between 4 and 6 mg and were scanned at 10 °C min^{-1}. The temperature range was 30–700 °C under a 60 mL min^{-1} flow rate of air (Samal et al. 2009; Samal et al. 2014; Samal et al. 2015).

2.5.In vitro biocompatibility evaluation of the samples using MTT cell viability assay

2.5.1. Cell culture and materials

Cytotoxicity evaluation of the samples was preformed according to the SR-EN IS 10993 Standard using the MTT (thiazolyl tetrazolium bromide) test, with cell morphology was revealed by Hematoxylin-Eosin staining and observed by optical microscopy. In experiment the cultured cells were exposure directly to the samples followed by cell viability MTT assay.

MTT tetrazolium cell proliferation assay as described for the first time by Mosmann in 1983, consists in a cellular reduction of the mitochondrial dehydrogenase enzyme by tetrazolium reagent in metabolically active cells and formation of a formazan precipitate, which requires the addition of a solubilizing agent to generate a solution suitable for recording absorbance. The amount of purple-blue solution obtained after dissolving in isopropanol is directly proportional to the number of viable cells in standard culture conditions.

In vitro biocompatibility assessing of INH and its derivatives based powder compounds was performed on Mouse fibroblasts (L929) cultured in T-25 tissue culture flasks and incubated overnight at 37 °C and 95% relative humidity in an air atmosphere containing 5% CO_2. The cell suspension was seeded at a density of 4×10^4 cells/mL and incubated in 24 well plates for 24 h until a monolayer was formed. For the extracts preparation of powder compounds, the samples were dissolved in a small amount of DMSO followed by solubilisation in the culture medium, obtaining solutions of samples of 1.75 mg / mL concentration, which were sterilised by filtration, before using in the experiment. After 24h of cells incubation in the 24 well plates, the medium from wells was replaced with 500 µL sample extract of powder compounds at 1.75 mg / mL concentration. In parallel with sample treatment, cells treated with hydrogen peroxide H_2O_2 (0.03%) were used as positive control, while cells untreated were used as culture control. All the samples and controls were tested in triplicate. The plates were incubated at 37 °C, and the quantitative evaluation of cytotoxicity was done after the desired time of samples exposure (24, 48 and 72 h) using tetrazolium salt (MTT) and the morphology of cells was assessed after 72h using an inverted microscope (Tihan et al. 2018).

2.5.2. MTT cell proliferation assay

For the MTT assay, the culture medium was replaced with fresh medium containing 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution, in a 10:1 (v/v) ratio and the plates were incubated at 37 °C, for 3 h. Then, 500 µL of isopropanol was added to each well to dissolve the formazan crystals by gently shaking on a platform, for 3 h. The optical density (OD) of the coloured solution was read at 570 nm using a Mithras LB940 microplates reader (Berthold, Germany). Cell viability was evaluated using the following formula:

$$\textit{Cell viability (\%)} = OD_{sample} / OD_{control} \times 100 \quad (1)$$

where: OD_{sample} = optical density of the sample and $OD_{control}$ = optical density of the untreated cell culture (Avwioro, 2011).

2.5.3. Hematoxylin-Eosin staining

The assayed cell cultures from well plates, after 72h of the experiment, were first washed with PBS, fixed with picric acid - formaldehyde (40%) (Sigma) solution, and stained with Hematoxylin and Eosin (Sigma) dyes (Vosátka et al., 2018). The cell morphology was observed and pictures were taken using an inverse-phase microscope (Nikon) and a photo camera AxioCam MRc.

2.6. In vitro antimicrobial assay

2.6.1. Absolute concentrations method

This test was performed for isoniazid and the three non-encapsulated derivatives, using as microbial strain, *Mycobacterium tuberculosis* ATCC 25177. Thus, dilutions of 1, 2 and 4 μg/ ml were used: for INH, the tested dilutions were 1 and 2 μg/ ml, as ideal concentration reported in literature (Agalloco and Carleton, 2007) and for INH-a, INH-b and INH-c, all three dilutions were tested.

0.5 ml of each dilution of the substances to be tested, were assigned in one tube. Then the tubes were placed in a horizontal position so that the solutions flooded the slope of the environment. They were kept in this position in the refrigerator for 48 hours, so the solutions were absorbed by the culture environment. Next, 0.2 ml of a standardized inoculum of the reference strain was dispensed into each tube. The tubes were incubated at 37º C, in a horizontal position for another 48 hours and then up to 28 days, in a vertical position. The reading was done by comparison between the control tube for which bacterial growth was assessed with *3+* (confluent culture) and the tubes with dilutions in DMSO.

2.6.2. Determination of the Minimum Inhibitory Concentration (MIC)

In order to check if the chemical changes made on the structure of the isoniazid have influenced the antimycobacterial activities of the new synthesized compounds, minimum inhibitory concentrations (MICs) against *Mycobacterium tuberculosis*, have been determined. For this, Microplate Almar Blue Assay (MABA) has been used (Franzblau et al., 1998) and adapted according to the technique described by the literature (Jhamb et al., 2014). Successive dilutions ranging from 25 to 0.012 μg/ mL of Isoniazid and the three tested derivatives (INH-a, INH-b and INH-c) were prepared directly in 96 well on the plate, in a volume of 50 μL of the Middlebrook 7H9 broth. Further, an inoculum of Mycobacterium tuberculosis (50 μL) was added in the wells, for each sample. Plates have been protected with parafilm and then incubated at 37º C for 8 days. After this period, 10 μL of fresh mixture (1:1 - Almar Blue 0.1

mg/ml reagent and 10% Tween 80) was added to the plate and again incubated for other 24 h at 37º C.

At the end of the experiment, the results were interpreted based on the coloration that appeared: blue color is specific for no bacterial growth, while the pink one indicates bacterial growth. Thus, the Minimum Inhibitory Concentration (MIC) was defined as the lowest drug concentration which prevents the appearance of pink coloration. Each assay was carried out in triplicate.

2.7.Toxicological screening

2.7.1. Determination of acute and chronic toxicity

Toxicology screening was carried out on white mice, male, Swiss breed weighing 25-30 g. The conduct of the study, including animal accommodation, handling, administration of test compounds and their slaughter, was carried out in accordance with current guidelines on the ethics of laboratory animal testing (Law No 206/27 May 2004, EU / 2010/63 - CE86 / 609 / EEC) and with the opinion of the UMF Research Ethics Commission "Grigore T. Popa" Iasi, issued on 17.04.2018.

Acute toxicity was assessed by the lethal dose assay 50, which consists in administering the compounds at geometric progression concentrations, to calculate the dose that kills 50% of the mice. The test compounds were administered in various concentrations (175-2000 mg/kg body weight) orally as suspensions in 1% CMC-Na solution and the survival rate was followed over a 24-hour period, 48 hours, 72 hours, 7 days and 14 days after administration.

For determining LD50, Karber's arithmetic method (Cheaburu Yilmaz et al. 2017) was used based on the following calculation formula:

$$LD50 = LD100 - \Sigma (a \times b) / n \qquad (2)$$

where,

a = the difference between two successive doses of the administered substance;

b = average number of dead animals in two successive batches;

n = number of animals in a lot;

LD100 = lethal dose 100 (representing the amount of substance that causes the death of all animals in the trial lot).

On the basis of the results obtained in the acute toxicity test, the chronic toxicity of the obtained compounds (INH, INH-a, INH-b and INH-c) was monitored by administering them at doses of 1/10 from LD 50, by oral gavage. Thus, the substances were dispersed in 1% CMC-Na solution and administered in a single dose, for 30 days. The animals were divided

into 6 groups (7 animals per group): groups 1 to 4 received suspensions with INH and its derivatives (INH-a, INH-b and INH-c); group 5 received the vehicle (1% CMC-Na solution) and group 6 has been preserved as an untreated control. At the end of the experiment, the mice were anesthetized by 100 mg/kg body weight, i.p. ketamine administration. After restraining by limb fixation, the chest was opened together with the abdominal area, for organ harvesting. The organs were fixed in 10% buffered formol, to be histopathologically analyzed.

2.7.2. Histopathological study

In order to evaluate the effect of INH derivatization, with various aromatic benzaldehydes on tissue toxicity, a histopathological study was performed firstly on fragments of the brain, cord, kidney, lung and liver, to identify morphological changes produced by the administration of lethal doses of the compounds, followed by a histopathological study on liver tissue fragments, to identify morphological changes produced by the chronic administration of the compounds.

The first step was to include tissue fragments taken in paraffin. The obtained paraffin blocks were cut into the microtome at 2-3 microns thick, the sections obtained being blown on the blades, after which they were specifically stained with H & E (Chiriac et al. 2017). The microscopic examination was performed using a Nikon Eclipse 50i microscope to see if there were any tissue alterations that highlighted the impact of acute and chronic administration.

2.8.Statistical Analysis

All assays were carried out in triplicate. Data were analysed by an analysis of variance (ANOVA) ($p < 0.05$) and were expressed as means ± SD (standard deviation) and significance was analysed using T-student test in Microsoft Excel forWindows.

3. RESULTS AND DISCUSSIONS

3.1.Physico-chemical and spectral characterization of new INH derivatives

Following optimization of the synthesis method, the condensation compounds of INH, like crystalline powders with coloring ranging from white to yellow (**Figure 2**), were obtained in very good yields, ranging from 83.52 to 95.73%. More than this, the melting points of these compounds ranged in maximum of 3 degrees, indicating their high degree of purity (**Table 1**).

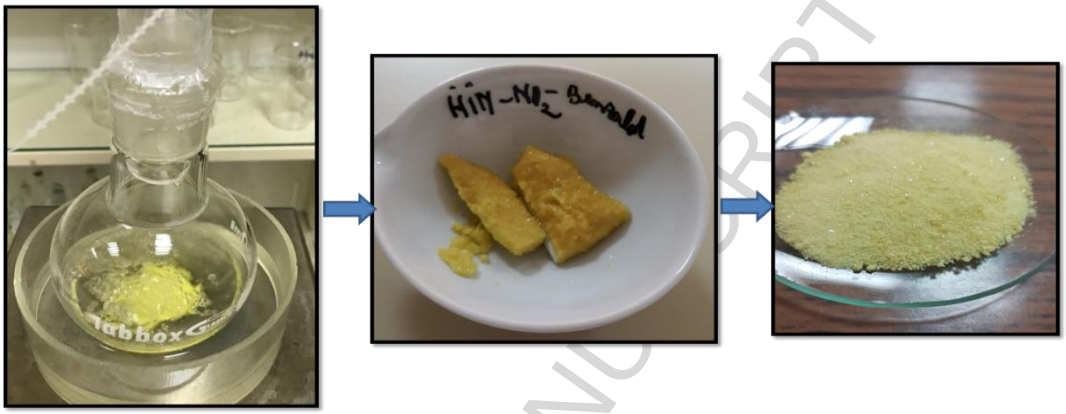


Figure 2. The steps of producing isoniazid derivatives (e.g., obtaining INH-b, the condensation product of isoniazid with 2-Nitrobenzaldehyde (b))

Table 1. Physico-chemical characterization of INH and its hydrazones

Compound	Aspect	Molecular formula	Molecular weight	Melting point (^{o}C)	η (%)
INH	white crystalline powder	$C_6H_7N_3O$	137,14	169-172	-
INH-a	white crystalline powder	$C_{12}H_{11}N_2O$	225,25	188-191	95,73
INH-b	yellow crystalline powder	$C_{12}H_{10}N_4O_2$	270,25	194	86,43
INH-c	crystalline white-yellowish powder	$C_{12}H_{10}N_2OBr$	304,15	205-207	83,52

Regarding the structural confirmation by spectral characterization, by analyzing the ATR-FTIR spectra of the hydrazones with isonicotinic structure (INH-a, INH-b, INH-c), obtained by treatment of isoniazid with various benzaldehydes, the absorption bands of all functional groups were identified, which confirms that condensation reactions have taken place.

Thus, in the ATR-FTIR spectrum of the three isonicotinic structure hydrazones (**Figure 3**), the azometin functional group (-N = CH-) was identified by the appearance of the high intensity absorption band, in the range 1551-1558 cm^{-1}. The occurrence of this band is an

argument for the condensation reaction between izoniazid and the aromatic aldehydes selected for this study (Benzaldehyde, 2-Nitrobenzaldehyde and 4-Bromobenzaldehyde). In addition to the azometin functional group, all other elements characteristic of the pyridine nucleus and the aromatic nucleus have been identified. The aromatic ring present in the aromatic aldehyde structure is found in the spectrum by the medium intensity bands, in the region of 1057-1065 cm^{-1}, due to the stretching vibrations of the CH bond and by the medium intensity bands in the range of 1589-1605 cm^{-1}, attributed to the stretching vibrations of the -C = C- bond. At the same time, the aromatic component used in the condensation reaction was also identified by the substituent in the 2 positions (in the case of INH-b derivative) and in the 4 positions (in the case of INH-c derivative). In the case of using 2-nitrobenzaldehyde, the presence of the nitro group (INH-b compound) is confirmed by the antisymmetric valence vibrations at 1350 cm^{-1} and, when 4-Bromobenzaldehyde is used in the condensation reaction, the presence of halogen bromine (compound INH-c) is confirmed at 559 cm^{-1}.

On the other hand, the pyridine nucleus of the isoniazid residue was identified by medium intensity absorption bands characteristic of the CH bond, in the region of 3009-3078 cm^{-1} and the amide –HNCO- group, in the range 1659-1674 cm^{-1}.

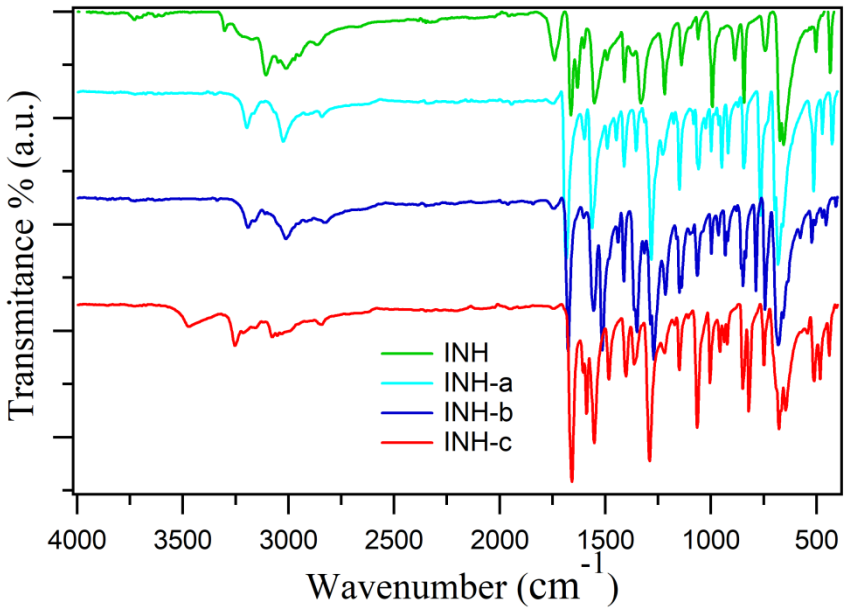


Figure 3. The IR spectra of INH and its derivatives (INH-a, INH-b, INH-c).

The NMR spectra of the hydrazones with isonicotinic structure, are found in **Figure 4**. The condensation of INH with aromatic benzaldehydes was confirmed by ^{1}H NMR spectroscopy. In the spectra of INH derivatives, the characteristic signals for both units: isoniazid and the aromatic ring of benzaldehyde have appeared, which demonstrates that condensation has

occurred. The spectra of the new derivatives (INH-a, INH-b and INH-c) showed slightly shifted signals attributed to the pyridine protons of INH (as compared with INH itself in Fig. 4), in the range 8.5-8,9 ppm. The aromatic protons present in the derivatives structure are found in the spectrum, in the range 7.33-8.33 ppm., which are attributed to totally absent signals from the isoniazid spectrum. In addition, the condensation proof is in the occurrence of CH=N group-specific proton, appeared as a singlet around δ 8.33- 8.67 ppm.

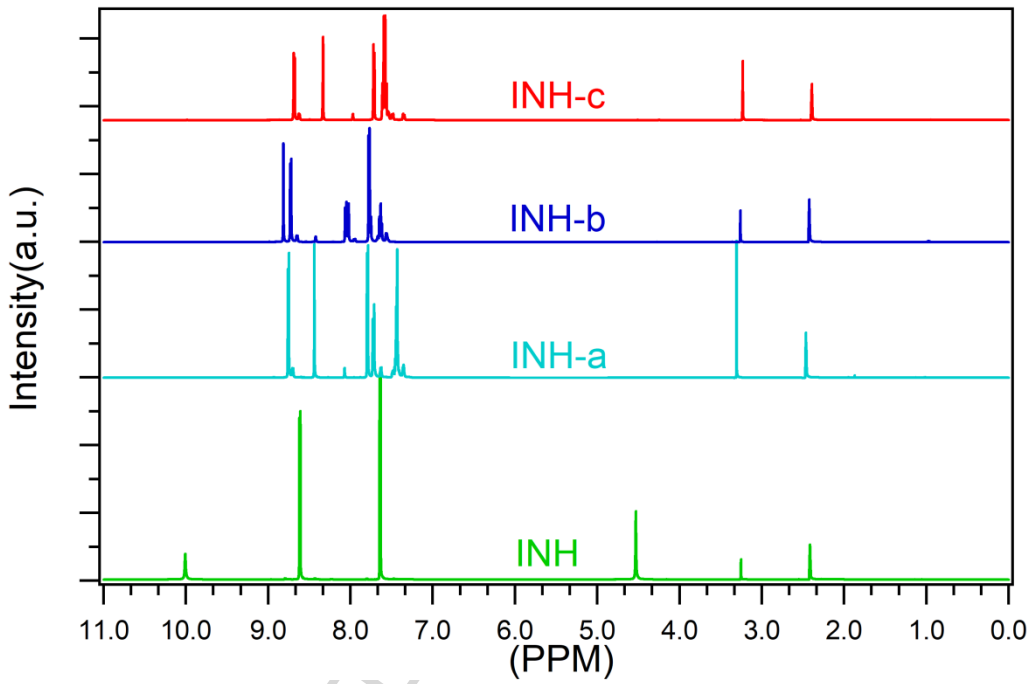


Figure 4. The NMR spectra of INH and its derivatives (INH-a, INH-b, INH-c).

3.2.X-ray diffraction study

Isoniazid and its derivatives were characterized further by XRD study. The X-ray diffraction patterns of new compounds are presented in **Figure 5**. The peak position (angle of diffraction) is an indication of the amorphous nature of the sample. The diffractogram of pure isoniazid shows some intense peaks, which indicates a higher crystallinity. In the case of its new derivatives, especially for INH-c (obtained by condensation with 4-Bromo-benzaldehyde), a reduced number of signals, of markedly low intensity, can be observed, indicating the greater amorphous nature (more water soluble), compared with parent isoniazid.

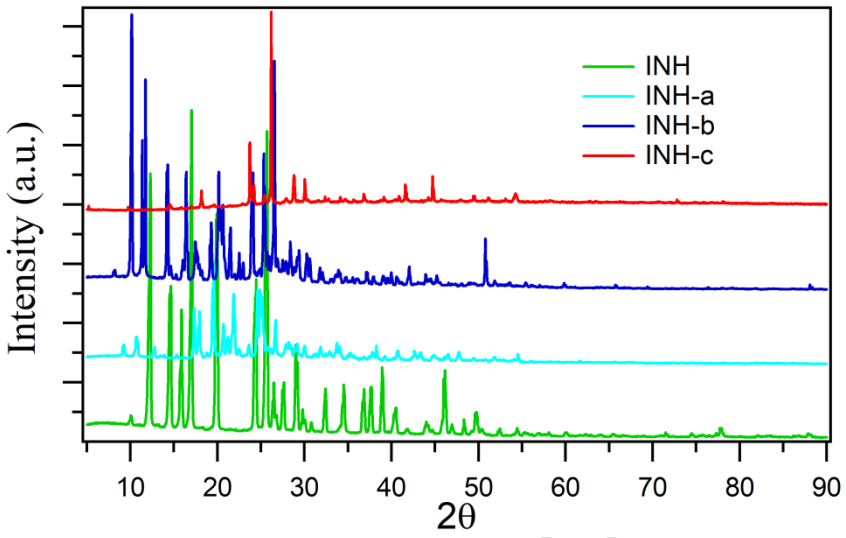


Figure 5. X-ray diffraction study on INH and its derivatives(INH-a, INH-b and INH-c).

3.3.Thermogravimetric Analysis (TGA)

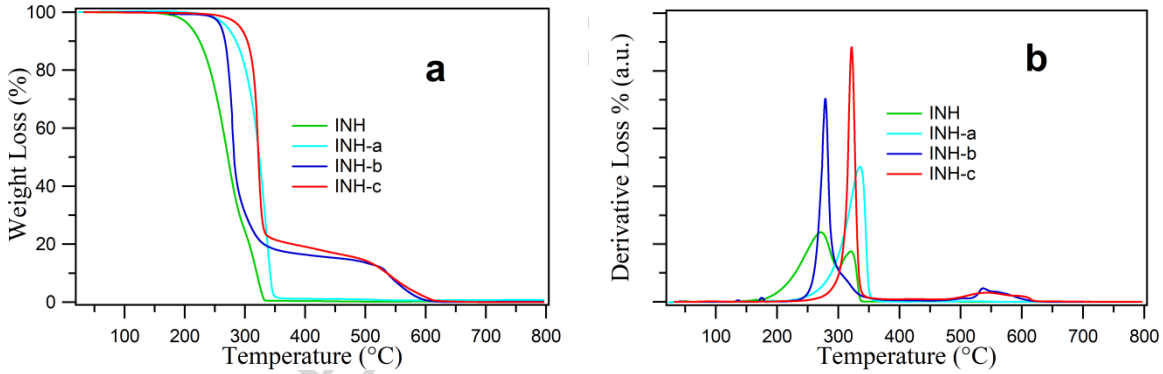


Figure 6. TGA spectra of INH and its derivatives (INH-a, INH-b, INH-c): **(a)** residual weight loss and **(b)** derivative weight loss at 10 °C min^{-1} with a temperature range of 30−800 °C under 60 mL min^{-1} flow rate of air.

The thermal stability of the INH and its derivatives were analysed by TGA. The TGA and derivative TGA spectra are depicted in **Figure 6a&b**. The traces of TGA show a continuous weight loss up to 800 °C. The first step loss of INH start at 185 °C and a small shoulder observed at 320 °C. The derivative curve of INH show maximum loss occurs at 270 °C and 320°C. The INH derivatives residual weight loss shows different degradation profiles from 200 to 800°C. The residual weight loss spectra demonstrate that the INH-b degradation start at 250 °C with sharp degradation up to 320 °C followed by shoulder at ~320-550 °C. INH-a start degradation around 260 °C and its residual weight become zero at 350 °C. However,

INH-c showed higher thermal stability compared to INH, INH-a and INH-b. Its degradation starts at 280 °C with sharp degradation up to 350 °C followed by a shoulder at ~350-550 °C. The derivative weight loss showed in **Figure 6b**, derivative loss peak of INH-a at 278 °C, INH-b at 335 °C and INH-c at 322 °C. In the case of INH-b and INH-c derivative spectra showed a plateau at ~ 500-600 °C. The derivative synthesized with 4-Bromo-benzaldehyde showed higher thermal stability.

3.4.In vitro biocompatibility evaluation of the samples using MTT cell viability assay

Table 2. The cell viability values (%) for tested samples of powder compounds at 24 h, 48 h and 72 h

	Cell viability (%)		
Samples	**24 h**	**48 h**	**72 h**
Control	100	100	100
H_2O_2 0.03% positive control	29.21	13.64	4.86
INH reference compound	84.02	55.15	46.04
INH-a	79.17	55.30	50.58
INH-b	87.73	61.16	53.14
INH-c	88.97	77.62	72.47

(where:80-100% is for non cytotoxic compunds; 50-80% is for slightly cytotoxic compounds; 30-50% for the moderately cytotoxic and < 30% for severe cytotoxic compounds) (ISO 10993-5, Geneva 2003)

In vitro biocompatibility evaluation by MTT assay in fibroblast cell line (NCTC clone L929) after 24h of contact with the samples, indicated that powder compounds at 1.75 mg / mL concentration, present good biocompatibility with NCTC cells. In this context, for samples INH-b and INH-c, 87.73% respectively 88.97% viability were recorded, higher than the INH reference compound (84.02%). After 48h and 72h of testing, the powder compounds especially INH-a and INH-b induced increasing cytotoxicity, with cell viability values between 55.30 % and 61.16 % (at 48 h), and 50.58 % and 53.14 % (at 72 h) , similar values as the INH reference compound (55.15 % at 48 h and 46.04 % at 72 h), all of them being within the limit of slightly to moderately cytotoxic compounds. The most noncytotoxic sample through all three intervals was INH-c with variable cell viability from 88.97% (at 24 h) to 72.47% (at 72 h), this one fitting so among non cytotoxic to slightly cytotoxic compounds (**Table 2**).

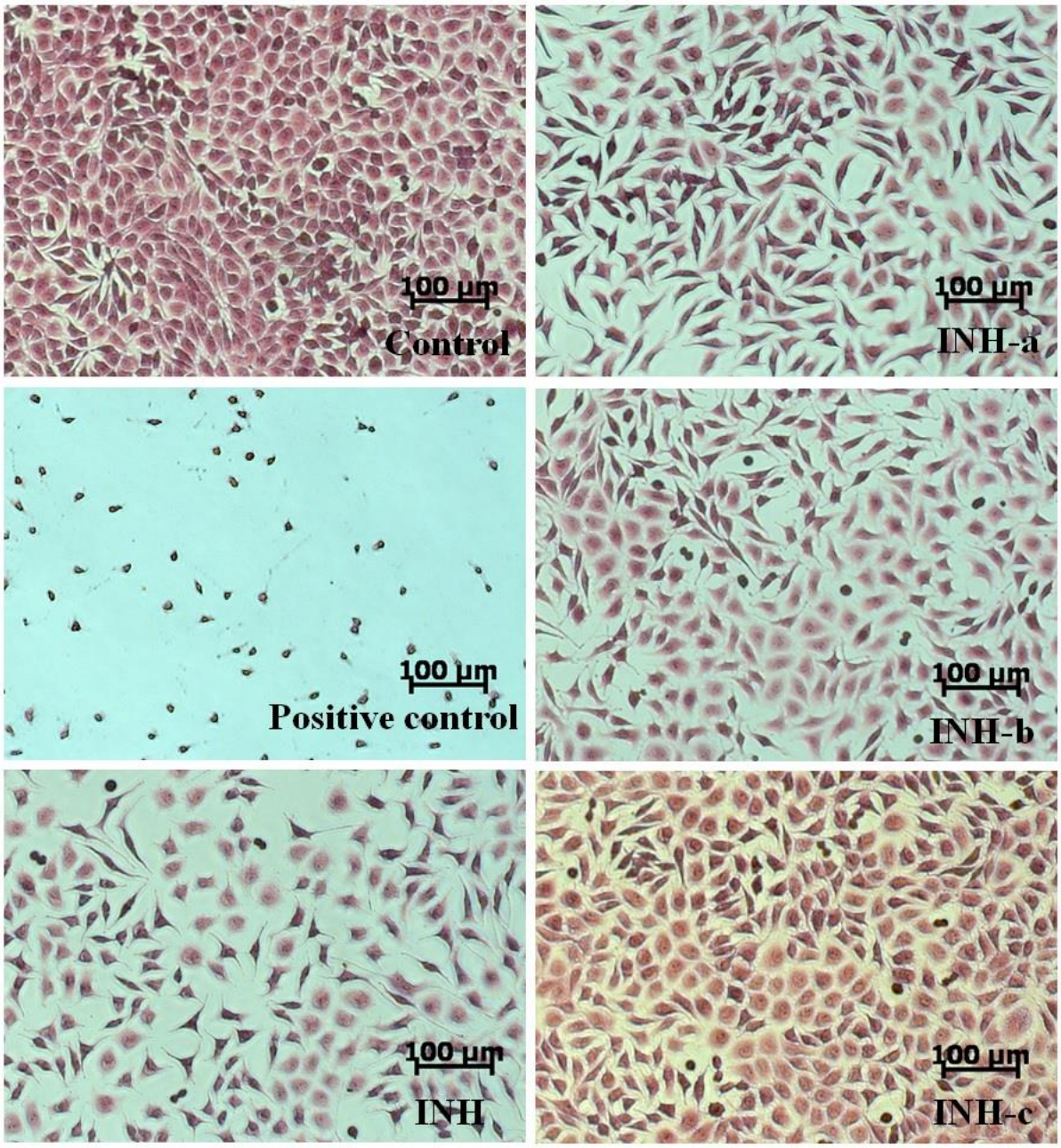


Figure 7. Cell morphology stained with Hematoxylin-Eosin after 72h of incubation with powders of INH derivatives and INH as reference compound.

Light microscopy images (**Figure 7**) of the cells morphology stained with Hematoxyline-Eosin after 72h of treatment with powders of INH derivatives, showed a normal morphology specific to untreated NCTC cells, with round and polygonal shape.

In addition, INH-c sample showed the most similar conformation and cell proliferation rate, which is comparable to the control culture. Cell morphology aspects of INH-c sampletreatment confirm the viability results obtained by MTT assay, suggesting good biocompatibility of this sample with NCTC fibroblasts.

3.5. *In vitro* antimicrobial assay

3.5.1. Absolute concentrations method

The results obtained for in vitro antimicrobial assay are presented in figure no. 8.

Figure 8. In vitro antimicrobial action of INH and its derivatives (INH-a, INH-b and INH-c).

Thus, from tube no. 1 to test tube no. 11 (1,2- INH; 3,4,5- INH-a; 6,7,8- INH-b; 9,10,11- INH-c) there has been no increase, which has led us to declare that the strain is sensitive to all tested dilutions (1, 2 and 4 μg/ ml). On the other hand, the DMSO solvent (tube no 23) allowed the growth of the tested bacterial strain, after 28 days of incubation.

3.5.2. Determination of the Minimum Inhibitory Concentration (MIC)

Table 3. In vitro activity of INH and 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

Even if the activity of isoniazid derivatives was found to be slightly lower than the parent isoniazid, their Minimum Inhibitory Concentrations fall within the required limits imposed by the Global Program for the Discovery of New Antituberculosis Drugs, namely 6.25 μg/mL as the upper limit for the evaluation of anti-tubercular activity of new therapeutic compounds (Matei et al. 2013).

In addition, their action is similar or even superior to that of other izoniazide derivatives mentioned in the literature: new thiocinnamamide-like thioamides as isoniazid derivatives with MIC values between 0.391 and 6.25 μg/mL (Matei et al. 2013); new isoziazid-azole

hybrides with MIC values ranging from 0.195 to 1.56 µM or new izoniazid-pyrrole hybride which was found active with MIC value of 3.2 µg/mL (Hu et al., 2017). Also, in their work, Castelo-Branco et al., 2018, have shown increased activity against *Mycobacterium tuberculosis* for new hydrazides derivatives of isoniazid with MICs of 3.59 and 6.91 µM, respectively.

In this study case, it is important to underline that the most active compound is INH-a (0.84 µg/mL), that is obtained by condensation with benzaldehyde, for which the aromatic ring is unsubstituted, while substitution of the aromatic ring with electron withdrawing groups (-NO_2 for INH-b and –Br for INH-c) has a negative influence on the activity, causing higher MIC values of 4.166 µg/mL and 1.785 µg/mL, respectively (table 3), but which fall within the above mentioned limit of 6.25 µg/mL.

In conclusion, all the compounds are active on the reference strain, *Mycobacterium tuberculosis* ATCC 25177, at various minimum inhibitory concentrations, which means that the condensation reaction with the three benzaldehydes, does not adversely affect the therapeutic effect of isoniazid.

3.6.Toxicological screening

3.6.1. Acute toxicity. LD 50 determination

Establishing LD 50 is an important step in assessing the toxicological profile of an active substance. The LD 50 values of the three derivatives as well as the isoniazid are shown in figure 9.

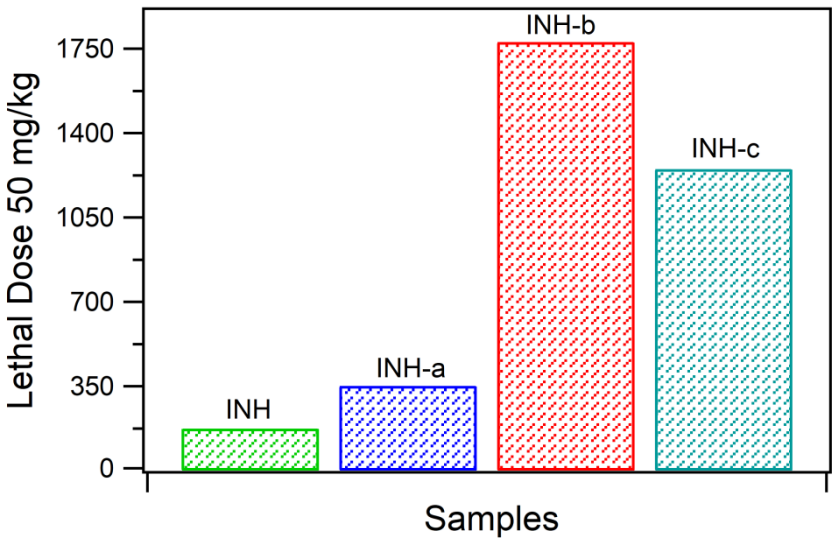


Figure 9. LD 50 values for INH and its derivatives INH-a, INH-b and INH-c

By analyzing the data presented in figure 9 it can be seen that the toxicity of INH-a, INH-b and INH-c derivatives is lower with LD50 values ranging from 352.34-1778.8 mg/kg body weight relative to isoniazid, the starting compound (INH) whose LD50 is 175.2 mg/kg body weight. Thus, compared to isoniazid, used as a structural model, the synthesized compounds, derivatives with hydrazone structure (INH-a, INH-b and INH-c), are up to about 10 times less toxic.

3.6.2. Histopathological study in the acute toxicity test

In the acute toxicity evaluation, fragments of the brain, cord, kidney, lung and liver were examined (**Figure 10**). Comparing the images of the control group with the group which was treated with the lethal dose of isoniazid, it was found that, at the cerebral, cardiac and hepatic levels, isoniazid did not cause any changes; instead, certain changes occur in the lung and kidney cases.

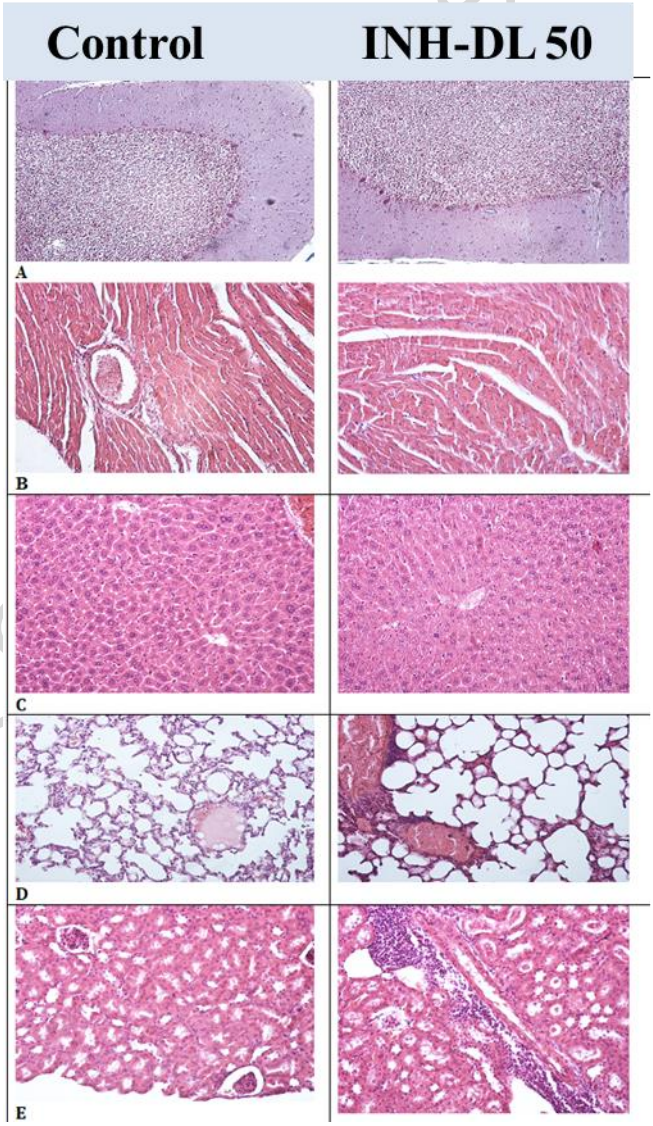


Figure 10. Histopathological study: brain (A), cord (B), liver (C), pulmonary (D), renal (E).

In the pulmonary area, the following pathological changes were microscopically revealed: alveolar spaces and alveolar walls destruction, vascular congestion, as well as inflammation areas. Renal, tubular necrosis accompanied by inflammatory areas that extend deep into renal parenchyma, mostly following the renal tubular track, have been reported. All this leads us to assert that animal death has occurred through respiratory and renal insufficiency.

3.6.3. Histopathological study in the chronic toxicity test

In order to evaluate the impact of the administered compounds on the tissues, liver fragments taken from all the groups under study (described in Material and Methods) were examined (**Figure 11**).

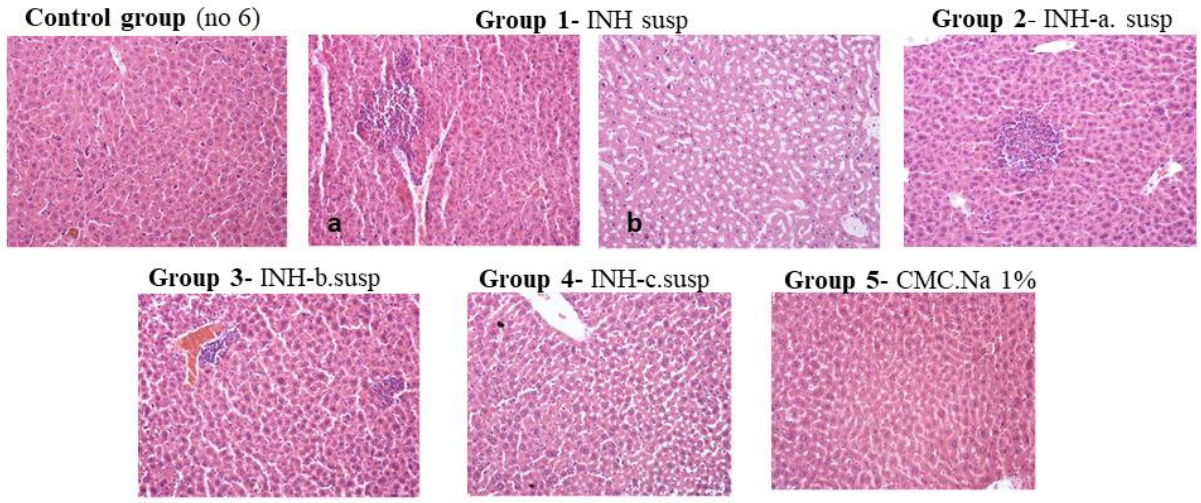


Figure 11. Histopathological study: fragments of liver tissues taken from animals that received INH and its derivatives in suspensions

The control group (no 6) revealed a normal hepatic morphology with hepatocyte cords radiating from the centrilobular vein and a normal parenchymal hepatic configuration.

Group 1, that received INH suspension, showed consistent liver damage: hepatic parenchyma has extensive areas of inflammation and cellular necrosis (**Figure11a**), but also well-defined areas of microvesicular steatosis (**Figure11b**), which attests the toxicity of isoniazid on liver tissue, well-known in the literature and presented in the introduction of this paper.

Group 2, that received INH-a suspension, also showed a sign of hepatic tissue suffering, manifested by frequent vascular congestion, diffuse inflammation, and the onset of cellular necrosis.

Group 3, that received INH-b suspension, revealed the presence of peri- and intrasinusoidal inflammation, capillary congestion and necrosis.

Group 4, that received INH-c suspension, showed the lowest signs of alteration of hepatic parenchyma compared to the other similar groups: only mild vascular congestion.

Group 5, that received 1% CMC-Na solution did not show changes in hepatic cytoarchitecton.

Comparing these groups between them, we can clearly see that the substance that produced the most significant tissue alterations, is INH, which shows in addition to the other derivatives, accentuated necrosis accompanied by microvesicular steatosis. At the opposite, INH-c is distinguished by normal-looking liver tissue with mild vascular congestion. In addition, a very important thing, is that these results of the in vivo test is consistent with the in vitro biocompatibility test, where INH-c also turned out to be the most noncytotoxic sample, with variable cell viability from 72.47% (at 72 h) to 88.97% (at 24 h).

4. CONCLUSIONS

By structural modulation of isoniazid, three isonicotinoylhydrazones have been obtained through condensation with various aromatic aldehydes in absolute ethyl alcohol, in order to reduce acute and chronic tissue toxicity. The structure of the synthesized compounds was confirmed by ATR-FTIR and RMN spectral analysis. ATR-FTIR spectra, recorded in the 500-4000 cm^{-1} range, revealed characteristic group vibrations for all structural elements specific to each compound: the pyridine nucleus, the aromatic nucleus and the condensation sign of the two components (the azometonic group). In the ^{1}H-NMR spectra of INH derivatives all the characteristic signals for isoniazid and the aromatic ring of benzaldehydes, have appeared, together with CH=N group-specific proton, which demonstrates that condensation has occurred.

In vitro biocompatibility evaluation, using MTT cell viability assay, revealed the most noncytotoxic sample, INH-c, while cell morphology aspects of this sample treatment, suggest a good biocompatibility of it. In addition, the condensation of isoniazid with the three aromatic benzaldehydes, resulted in the significant reduction of acute toxicity, by lethal doses up to ten times higher. Furthermore, the disappearance of microvesicular steatosis areas was noted, in the chronic toxicity test.

Even if antimicrobial activity decreased with the introduction of electron withdrawing groups, at positions 2 and 4 of benzaldehyde structure used, by recording higher MIC values of new derivatives, this happens concurrently with significant reduction in toxicity of these compounds. From this point of view, the most active compound was INH-a, obtained by condensation of isoniazide with benzaldehyde.

All these results lead us to conclude that the objective of the study has been successfully completed, respectively, new isoniazid derivatives with improved pharmacotoxicological profile were obtained. In the future, we aim to reduce the appearance of micro-vesicular steatosis, produced in case of chronic administration with isoniazid, by using encapsulation process with chitosan, which is well known for its hypolipidemic properties (Dragostin I., et al., 2017). In conclusion, the obtained isonicotinoylhydrazones, from the study, are original and with the potential biological application as antimicrobial agents, in the treatment of tuberculosis.

ACKNOWLEDGMENTS

Funding: „This work was supported by a grant of Ministry of Research and Innovation, CNCS - UEFISCDI, project number PN-III-P1-1.1-PD-2016-0233, within PNCDI III. (Contract No. PD 144/2018)."

REFERENCES

Abdel-Aziz, M., Abdel-Rahman, H.M., 2010. Synthesis and anti-mycobacterial evaluation of some pyrazine-2-carboxylic acid hydrazide derivatives. *Eur. J. Med. Chem.* 45, 3384-3388.

Abdelrahman, M.A., 2017. Design, synthesis and 2D QSAR study of novel pyridine and quinolone hydrazone derivatives as potential antimicrobial and antitubercular agents. *Eur. J. Med. Chem*. 138, 698-714.

Agalloco, J. P., Carleton, F.J., 2007. Validation of Pharmaceutical Processes, 3rd ed.; CRC Press: Boca Raton, FL, USA,.

Ahmad, Z., Sharma, S., Khuller, G., 2005. In vitro and ex vivo antimy-cobacterial potential of azole drugs against Mycobacterium tuberculosis H37Rv. *FEMS Microbiol. Lett.* 251, 19-23.

Avwioro, G., 2011. Histochemical Uses of Haematoxylin - A Review Godwin Avwioro, *JPCS.* 1, 24-34.

Bartzatt, R., Cirillo, S., Cirillo, J.D., 2012. Small molecule hydrazide agents to inhibit growth and proliferation of Mycobacterium tuberculosis. *Med. Chem.* 8, 273-280.

Carroll, M.W., et al., 2013. Efficacy and safety of metronidazole forpulmonary multidrug-resistant tuberculosis. *Antimicrob. Agents Chemother.* 57, 3903-3934.

Carroll, P., Parish, T., 2015. Deletion of cyp125 Confers increased sen-sitivity to azoles in Mycobacterium tuberculosis. *PLOS ONE.* 10, e0133129.

Carvalho, S.A., et al., 2008. Synthesis and antimycobacterial evaluation of new trans-cinnamic acid hydrazide derivatives. *Bioorg. Med. Chem. Lett.* 18, 538-541.

Castelo-Branco FD., et al., 2018. New hydrazides derivatives of isoniazid against Mycobacterium tuberculosis: Higher potency and lower hepatocytotoxicity. *Eur. J. Med. Chem*. 146: 529-540.

Cheaburu Yilmaz, C. N., et al., 2017. Toxicity, Biocompatibility, pH-Responsiveness and Methotrexate Release from PVA/Hyaluronic Acid Cryogels for Psoriasis Therapy, *Polymers.* 123: 9, 1-14.

Chiriac, A. P., et al., 2017. The influence of excipients on physical and pharmaceutical properties of oral lyophilisates containing a pregabalin-acetaminophen combination. *Expert Opin. Drug Del.* 1-11.

Da Silva Lourenço, M.C., et al., 2008. Synthesis and anti-mycobacterial activity of (E)-N0-(monosubstituted-benzylidene) isonicotinohydrazide derivatives. *Eur. J. Med. Chem.* 43, 1344-1347.

Dragostin I., et al., 2017. The importance of polymers for encapsulation process and for enhanced cellular functions. *J Macromol Sci A.* 54(7): 489-493

Franzblau SG., et al., 1998. Rapid, lowtechnology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. *J. Clin. Microbiol.* 36: 362-366.

Global tuberculosis report 2018. (Geneva, World Health Organization, 2018. Licence: CC BY-NC-SA 3.0 IGO.,

Hoagland D.T., et al., 2016. New agents for the treatment of drug-resistant Mycobacterium tuberculosis. *Adv. Drug. Deliv. Rev.*. 102: 55–72.

Hossein, K. J., et al., 2017. Protective effects of salep against isoniazid liver toxicity in wistar rats. *J. Tradit. Complement. Med.* 8, 239-240.

Hu Y-Q., et al., 2017. Isoniazid derivatives and their anti-tubercular activity. *Eur. J. Med. Chem*. 133: 255-267.

Iona, E, et al., 2007. Metronidazole plus rifampin sterilizes long-term dormantM. Tuberculosis. *Antimicrob. Agents Chemother.* 51, 1537-1544.

ISO 10993-5. Biological Evaluation of Medical Devices – Part 5: Tests for Cytotoxicity: In Vitro-Methods, International standard, Geneva 2003.

Jhamb SS., et al. 2014. Determination of the activity of standard anti-tuberculosis drugs against intramacrophage Mycobacterium tuberculosis, in vitro: MGIT 960 as a viable alternative for BACTEC 460. *J. Infect. Dis.* 18 (3):336–340.

Loscalzo, J., Harrison, J. Pneumologie şi terapie intensivă, 2017, Ed All, Bucuresti;

Maccari, R., Ottana, R., Vigorita, M.G., 2005. In vitro advanced antimycobacterial screening of isoniazid-related hydrazones, hydrazides and cyanoboranes. *Bioorg. Med. Chem. Lett.* 15, 2509-2513.

Manetti, F., et al., 2000. Building a pharmacophore model for a novel class of antitubercular compounds. *Il Farm.* 55, 484-491.

Matei L., et al. 2013. Synthesis and bioevaluation of some new isoniazid derivatives. *Bioorg. Med. Chem.* 21: 5355-5361.

Navarrete-Vazquez, G., et al., 2007. Synthesis and antimycobacterial activity of 4-(5-substituted-1,3,4-oxadiazol-2-yl) pyridines. *Bioorg. Med. Chem.* 15, 5502-5508.

Piccaro, G., et al., 2013. Activities of drug combinations against Mycobacterium tubercu-losis grown in aerobic and hypoxic acidic conditions. *Antimicrob. Agents. Chemother.* 57, 1428-1433.

Samal, S.K. etal., 2009. Thermal Analysis of 2D PVA/CNTs Membrane, *J. Therm. Anal. Calorim.*, 97, 859 – 864.

Samal, S.K. et al. 2014., Enzymatic Mineralization of Silk Scaffolds, Macromol. Biosci., 14, 991-1003.

Samal, S.K., et al. 2014., Silk/chitosan biohybrid hydrogels and scaffolds via green technology, *RSC Adv.*, 4, 53547 – 53556.

Samal, S.K., etal. 2015., Multilayered Magnetic Gelatin Membrane Scaffolds. *ACS Appl. Mater. Interfaces*, 7, 23098-23109.

Samal, S.K., etal. 2015., Biomimetic Magnetic Silk Scaffolds, *ACS Appl. Mater. Interfaces*, 7, 6282-6292.

Silva, F.P., et al., 2006. Experimental and theoretical structure characterization of two isoniazid derivatives: 2,4-Difluoro-N0-isonicotinoylbenzohydrazide and 2,4-dichloro-N0-isonicotinoylbenzohydrazide hydrochloride. *J. Mol. Struct.* 788, 63-71.

Sriram, D., Yogeeswari, P., Madhu, K., 2005. Synthesis and in vitro and in vivo antimycobacterial activity of isonicotinoyl hydrazones. *Bioorg. Med. Chem. Lett.* 15, 4502–4505.

Susmita, S., Advaita, G., Hoon, H. S., 2016. Current Overview of Anti-Tuberculosis Drugs: Metabolism and Toxicities. *Mycobact Dis.* 6, 2-5.

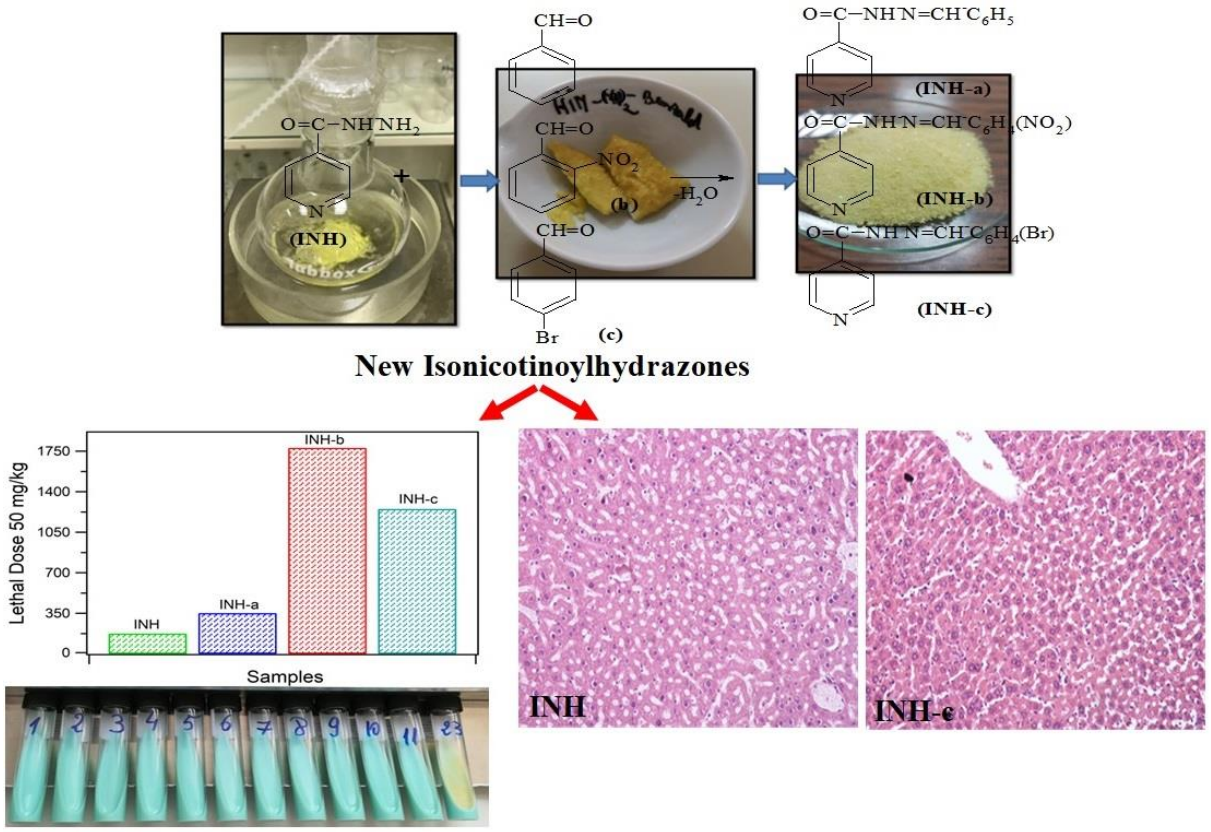
Talip B.A., et al., 2013. An Update on Global Tuberculosis (TB). *Infectious Diseases: Research and Treatment*. 6: 39–50.

Tihan, G. T. et al., 2018. Alkaline Phosphatase Immobilization on New Chitosan Membranes with Mg^{2+} for Biomedical Applications. *Mar. Drugs.* 287:16, 1-14.

Vidyasagar, R., Guruprasad, P., 2012. A Hepatotoxicity Related to Anti-tuberculosis Drugs: Mechanisms and Management. *J. Clin. Exp. Hepatol.* 7, 38-44.

Vosátka, R., et al., 2018. New lipophilic isoniazid derivatives and their 1,3,4-oxadiazole analogues: Synthesis, antimycobacterial activity and investigation of their mechanism of action. *Eur. J. Med. Chem.* 151, 824-835.

Yates, T.A., et al., 2016. The transmission of Mycobacterium tuberculosis in high burden settings. *Lancet Infect Dis.* 16, 227–238.



Graphical abstract