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Hepatoprotective agent tethered isoniazid for the treatment of drug-induced hepatotoxicity: Synthesis, biochemical and histopathological evaluation

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Triethylamine (PubChem CID: 8471)
4-Dimethylaminopyridine (PubChem CID: 14284)
Succinic Acid (PubChem CID: 1110)
Succinic Anhydride (PubChem CID: 7922)
Citric Acid (PubChem CID: 31348)

ABSTRACT

The aim of the study was to investigate the protective effect of isoniazid–curcumin conjugate (INH–CRM) in INH-induced hepatic injury by biochemical analysis and histology examination of liver in Wistar rats. The biochemical analysis included determination of the levels of plasma cholesterol, triglycerides (TG), albumin content, and lipid peroxidation (MDA). INH–CRM administration resulted in a significant decrease in plasma cholesterol, TG, and MDA levels in the liver tissue homogenate with an elevation in albumin level indicating its hepatoprotective activity. Histology of the liver further confirmed the reduction in hepatic injury. The hepatoprotective with INH–CRM can be attributed to the antioxidant activity of curcumin. The conjugate probably stabilizes the curcumin molecule, preventing its presystemic metabolism thereby enhancing its bioavailability and therefore, its hepatoprotective activity. Thus, the novel INH–CRM has the potential to alleviate INH-induced liver toxicity in antitubercular treatment.
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1. Introduction

Tuberculosis (TB) is a contagious disease which is a major challenge to public health. After AIDS, TB is the second most infectious disease [1,2] caused by the intracellular parasite Mycobacterium tuberculosis (MTB). The standard treatment consists of first-line anti-TB drugs (ATDs) viz., rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (ETH). The administration of INH, one of the mainstream antitubercular drug results in many metabolic and morphological aberrations in the liver, which is its main detoxifying site. INH after metabolism in the liver produces hydrazine metabolites (nitrogen-centered free radicals). These radicals generate highly reactive oxygen

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species, which act as stimulator of lipid peroxidation resulting in cell death and hepatic necrosis [3].

Phenolic substances have received much attention due to their biological activities and antioxidant properties [4]. Curcumin (CRM), 1,7-Bis(4-hydroxy-3-methoyphenyl)-1,6-heptadiene-3,5-dione, is a typical representative that exhibits powerful antioxidant effects in biological systems, including free radical scavenging and metal ion sequestering [5–7]. CRM has exhibited antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic, and hepatoprotective activities [8]. However, CRM exhibits poor oral bioavailability which is associated with its poor solubility and extensive presystemic and systemic metabolism [9]. Among the methods adopted to enhance its oral bioavailability, conjugation has been widely explored. Conjugation with water-soluble moieties has been reported to increase CRM solubility and decrease the extent of its presystemic metabolism [10].

It is used in the food and pharmaceutical industry as a stabilizer, natural colorant, and substitute for artificial colorants in cheese, soup, pickle, etc.

Various attempts have been made to overcome the antitubercular drug induced toxicity. They include conjugation with peptide, polyethylene-glycol and gelatin [11–13]. The aim of this study was the synthesis of a novel isoniazid–curcumin conjugate (INH–CRM) for improved toxicity profile of INH. INH–CRM formation was confirmed by mass spectroscopy and NMR. Further, the toxicity of the conjugate was compared with INH by biochemical analysis and histopathology. INH–CRM demonstrated significant improvement in hepatoprotective activity indicating its potential in tubercular therapy.

2. Materials and methods

2.1. Materials

INH was purchased from All Well Pharma, Chandigarh, India. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was bought from HiMedia Laboratories Pvt. Ltd. Mumbai, India. CRM (95%), Dicyclohexylcarbodiimide (DCC), Triethylamine (TEA) and 4-(dimethylamino) pyridine (DMAP), succinic acid, and succinic anhydride were bought from Sigma-Aldrich (Missouri, USA). Citric acid was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All other solvents were of analytical grade.

2.2. Synthesis of isoniazid–curcumin conjugate

An intermediate was synthesized by treating INH (10 mMol) with aqueous glyoxylic acid monohydrate (10 mMol) solution. The intermediate was mixed with CRM (equimolar amounts) in DCM and stirred constantly for thirty minutes at room temperature in a nitrogen atmosphere. Then DMAP was added in the catalytic amount of 8 mol % followed by EDAC after 15 min. Reaction was continued for 12 h to yield INH–CRM.

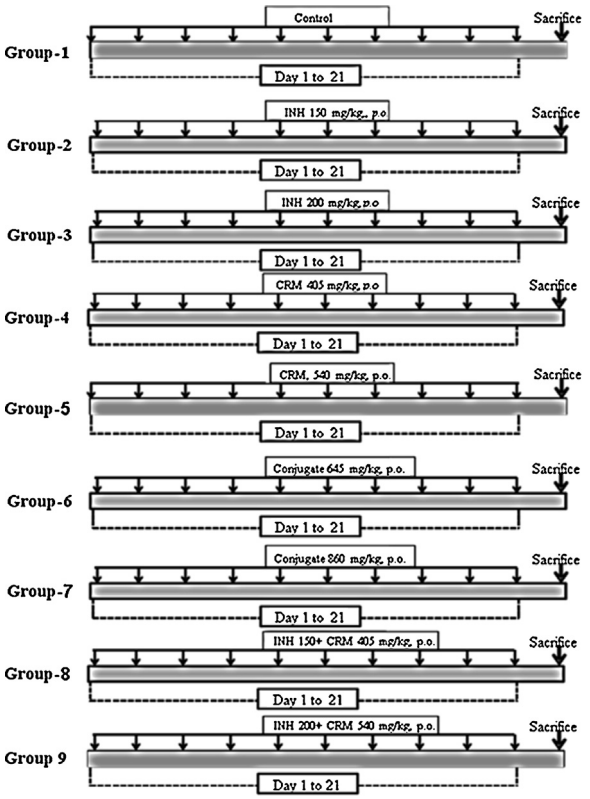

Fig. 1. Experimental design of the animal studies.

2.3. Characterizations of INH–CRM conjugate

2.3.1. NMR

The NMR of INH, CRM, intermediate, and INH–CRM was obtained from Bruker 400 UltraShield^{TM} (Bruker, Germany) spectrometer. The data were processed and analyzed with Topspin software.

2.3.2. Mass spectroscopy

To confirm the formation of INH–CRM conjugate, samples were dissolved in methanol then filtered and analyzed by mass spectroscopy (LCQ, Finnigan, MAT system equipped with Xcaliber software).

2.4. In vivo study for hepatoprotective activity

All animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC No. 108/1999/CPCSEA) and studies were performed in accordance with the CPCSEA (Committee for Purpose of Control and Supervision of Experimentation on Animals) guidelines. Wistar rats (180–200 g) were procured from the Central Animal Facility (CAF) of the institute. All the animals were maintained under controlled environmental conditions at room temperature ($22 \pm 2\,^{\circ}C$) with $50 \pm 10\%$ RH. Animals were acclimatized for 1 week prior to experimentation.

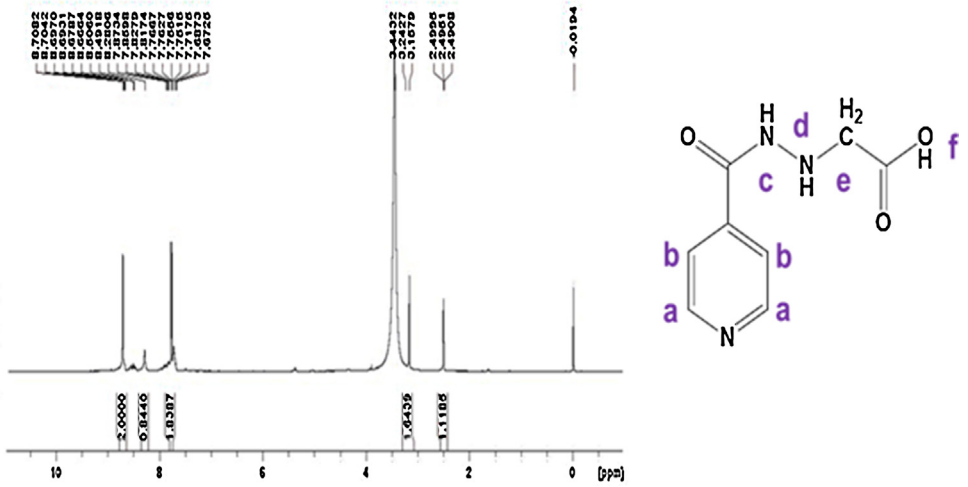

Fig. 2. NMR spectra of intermediate.

2.4.1. Experimental design

The study was conducted for 21 days. Animals were divided into nine groups (Fig. 1). There is considerable variation in INH and CRM doses for studies. Dose selection was done purely on the basis of reports available in the literature for INH [14,15] and CRM [16–20], respectively. Group I formed the negative control. Groups II and III were administered INH at 150 and 200 mg/kg/day doses, respectively. Groups IV and V were administered CRM at 405 and 540 mg/kg/day doses, respectively. Groups VI and VII were administered INH–CRM at 645 and 860 mg/kg/day doses. Groups VIII and IX were administered combination of free INH (150 and 200 mg/kg/day) and CRM (405 and 540 mg/kg/day), respectively. The hepatoprotective effect

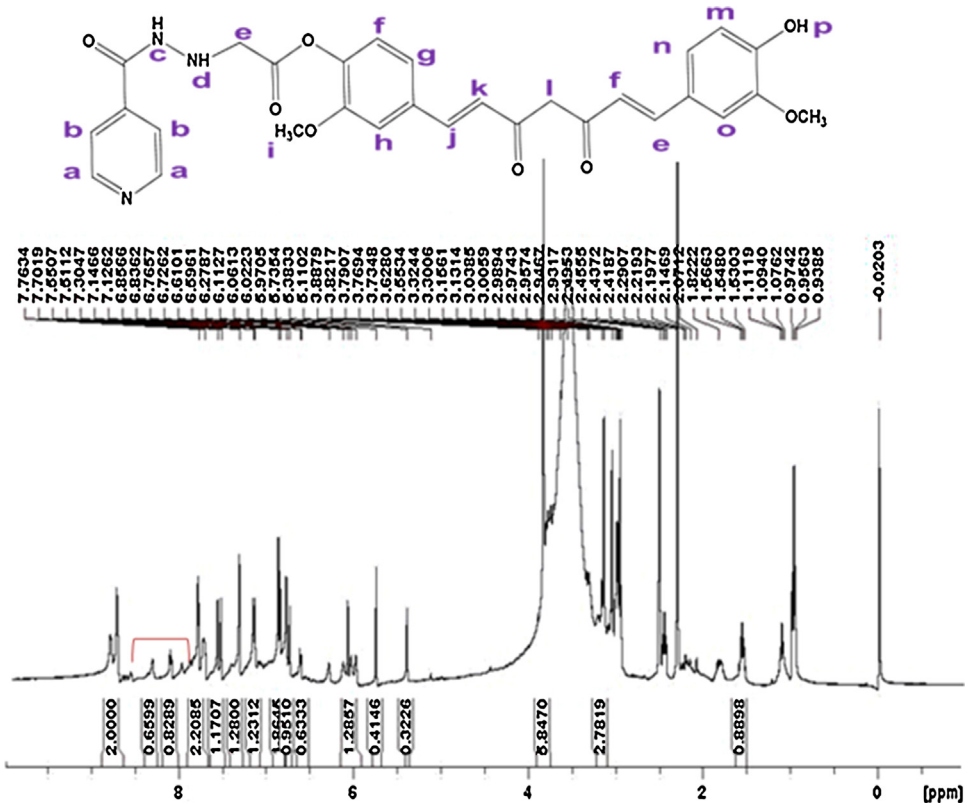

Fig. 3. NMR spectra of conjugate.

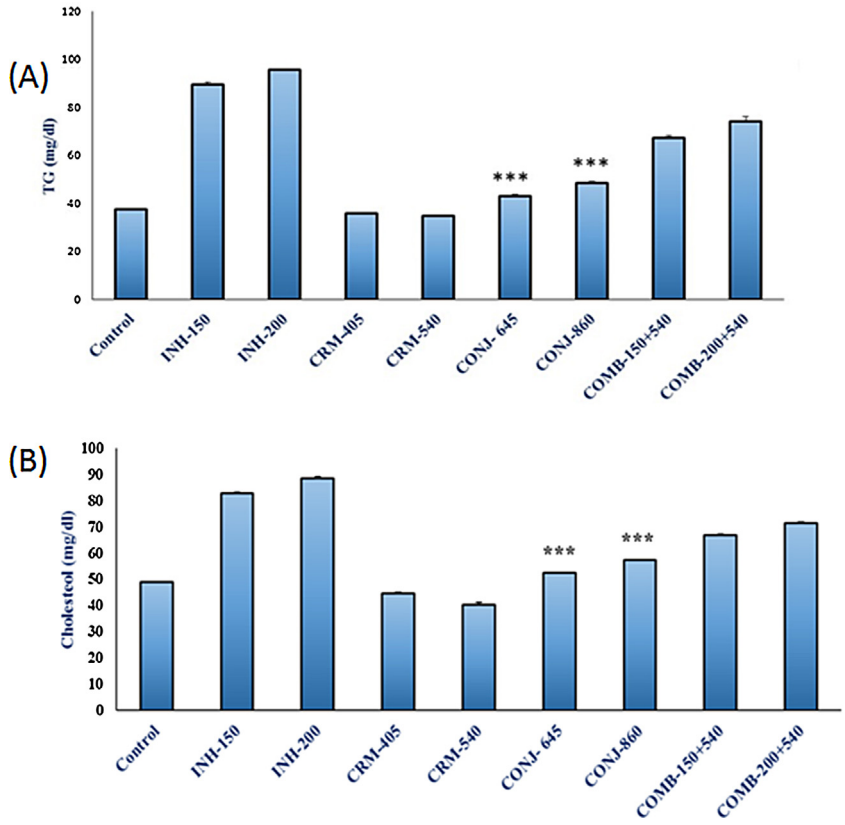

Fig. 4. (A) Effect of INH–CRM on plasma TG levels; (B) plasma cholesterol levels at the end of 3 weeks. INH–CRM was given p.o/day during 3 weeks. All values are expressed as mean ± SEM ($n = 4$), ***$p < 0.001$ vs INH-200.

of CRM was compared with the conjugate. Each experimental group was containing four animals for the study.

3. Toxicity profile

3.1. Biochemical parameters

The triglyceride and cholesterol levels were measured with spectrophotometric kits (Accurex Biomedical Pvt. Ltd., Mumbai, Maharashtra, India). Briefly, blood samples (approximately 0.6 mL) were collected from the retro-orbital plexus under light ether anesthesia in heparinized microcentrifuge tubes. Plasma was separated by centrifugation at 2500 × *g* for 5 min and analyzed.

3.2. Measurement of lipid peroxidation

Lipid peroxidation was quantified by the thiobarbituric acid reactive substance method, with some modifications. The level of malondialdehyde (MDA), the end product of lipid peroxidation is measured by this method. In brief, tissues were collected and homogenized in an ice cold phosphate buffer (pH 7.4). After centrifugation, the supernatant was collected and MDA levels were measured. Lipid peroxidation was calculated from the standard curve of 1, 1, 3, 3-tetramethoxy propane (97%) and expressed as nmol MDA/mg of protein [21].

3.3. Determination of protein content

Protein concentration in the tissue homogenate was determined by utilizing bovine serum albumin (BSA) (Sigma-Aldrich, USA) as the standard protein.

3.4. Histopathological evaluation

The morphological changes in the hepatic tissue were assessed by histopathology. Tissue sections were fixed in 10% formalin solution, dehydrated in increasing concentrations of ethanol followed by engrafting in paraffin. The sections were mounted on glass slides coated with Mayer's albumin and dried overnight. They were de-paraffinized with xylene, rehydrated with alcohol, and water mixture. The rehydrated sections were stained with hematoxylin and eosin, mounted with DPX media and viewed under the microscope at both high (40×) and low (10× and 20×) magnifications (Olympus BX51 microscope, Tokyo, Japan) [22].

3.5. Statistical analysis

Results are presented as mean ± standard error of mean (SEM) for each group. Statistical analysis was performed using Jandel Sigma Stat (Version 3.5) statistical software. Significance of the difference between two groups was evaluated using Student's *t*-test. For multiple comparisons,

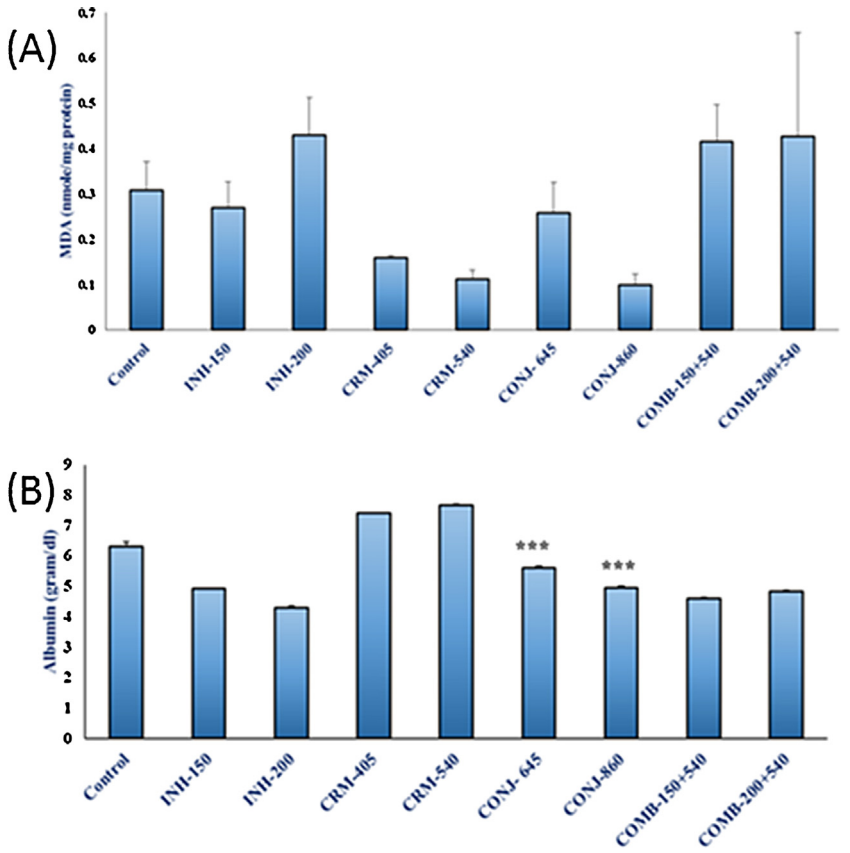

Fig. 5. (A) Effect of INH–CRM on liver MDA levels; (B) plasma albumin levels at the end of 3 weeks. INH–CRM was given p.o/day during 3 weeks. All values are expressed as mean ± SEM ($n = 4$) ***$p < 0.001$ vs INH-200.

one-way analysis of variance (ANOVA) was employed. When ANOVA exhibited significant difference, post-hoc analysis was performed with Tukey's test. $P < 0.05$ was considered to be statistically significant.

4. Results

4.1. Synthesis of INH–CRM

The conjugate was composed of 1:1 molar ratio of CRM and INH. The fraction of

CRM was about 0.728 while INH was 0.272 in the INH–CRM. Synthesis of INH–CRM was through an intermediate compound which was then reacted with CRM to obtain INH–CRM.

4.2. Characterization of INH–CRM

4.2.1. Nuclear magnetic resonance

NMR spectrum of the intermediate (Fig. 2) exhibits a characteristic proton peak at 2.5 ppm indicating the "d" proton of the intermediate. INH–CRM (Fig. 3) presented a chemical shift at 1.53 ppm corresponding to "d" proton, which is absent in CRM. In addition, the characteristic peaks at 8.81, 8.34, and 8.19 ppm in the conjugate indicate the presence of "a," "b," and "c" protons, respectively, confirms INH–CRM formation.

4.2.2. Mass spectra

The MS spectra of INH–CRM revealed the formation of INH–CRM exhibiting m/z peak at 545.11 while intermediate's peak was at m/z 195 (Data not presented). Hence, results of NMR and mass spectra support each other in confirming INH–CRM formation.

4.3. Animal studies

4.3.1. Biochemical assays

Biochemical tests indicating hepatocellular integrity were analyzed. Body weights increased in all the groups at the end of the study with no significant difference between the groups. Mortality rate (25%) was observed during entire study protocol from INH 200 mg/kg group. Similar results are reported in earlier studies [23]. INH administration resulted in a significant elevation in plasma triglycerides, cholesterol, and malondialdehyde (MDA) levels indicating its impact on integrity of hepatocytes with significant decrease in albumin levels [5]. The decrease in albumin level indicates the effect of INH on liver function.

In the INH–CRM group, TG level decreased significantly ($p < 0.001$) in comparison to the group treated with INH (dose of 200 mg/kg/day) thus demonstrating its hepatoprotective activity. TG level decrease, to a lesser extent, in the group treated with combination of INH and CRM and in

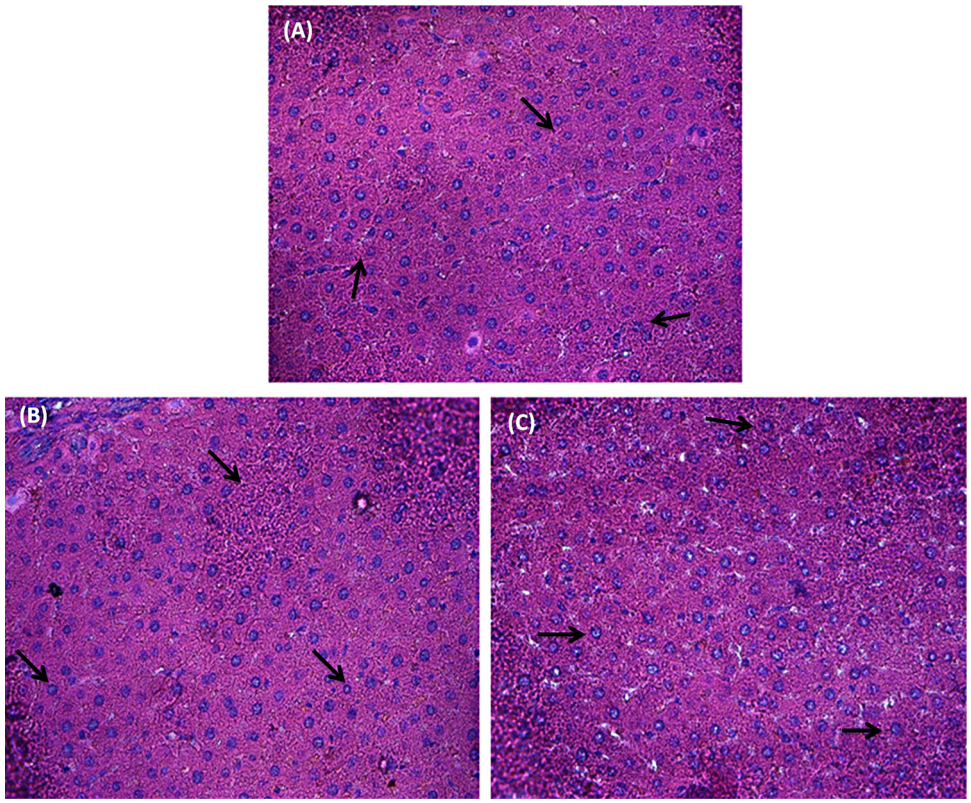

Fig. 6. Histological section of control group (A); histological section of groups treated with CRM 405 mg/kg/day (B); and CRM 540 mg/kg/day (C).

the CRM group. This decrease in plasma TG level can be attributed to the antioxidant effect of CRM (Fig. 4A).

Santhosh et al. [24] reported an increase in cholesterol level due to the devastating effect of hepatotoxic agents which disrupt membrane fluidity thereby affecting its functions. In agreement with this study, we observed that altered levels of plasma cholesterol in INH-treated rats in comparison to the control and INH–CRM-treated groups. Plasma cholesterol level was significantly ($p < 0.001$) reduced in INH–CRM-treated group in comparison to INH (200 mg/kg/day) group demonstrating hepato-protection of INH–CRM. In the INH and CRM combination group, the level of plasma cholesterol was less than in INH group. Thus, plasma cholesterol studies revealed that INH–CRM offered a significant protection against INH-induced alteration in the plasma cholesterol level. This observation can be attributed to CRM antioxidant properties (Fig. 4B).

MDA is a free radical which increases in hepatotoxic conditions. Its concentration is an index of lipid peroxidation. Some studies have also reported the thiobarbituric acid-reactive substances (TBARS) levels as an index of MDA [25–28]. Fig. 5A indicates significant ($p < 0.001$) decrease in MDA levels in the liver tissue homogenate of INH–CRM treated vs INH-treated group. Although, the group administered combination of INH and CRM exhibited low MDA level in comparison to free INH, the decrease was substantially less than the conjugate group. The decreased MDA formations following INH–CRM treatment confirm the hepatoprotective and antioxidant properties of CRM, as reported in previous studies [18,29,30].

Hepatic damage influences normal cellular metabolism [31]. Liver synthesizes albumin and a low serum albumin level indicates poor liver function suggesting liver disease [32]. The level of albumin significantly ($p < 0.001$) (Fig. 5B) increased in INH–CRM groups more than in case of combination of INH and CRM or CRM alone, thus confirming its effectiveness.

Hence, results of the biochemical evaluations indicate INH–CRM potential in alleviating the drug-induced hepatotoxicity. The enhanced activity of the conjugate can be assigned to its effect in reducing the presystemic metabolism of CRM by steric hindrance [33].

4.3.2. Histopathological evaluation

Rats have been successfully employed as models to investigate INH-induced hepatotoxicity. In the present study, INH was orally administered for 21 days to induce hepatoxicity in the test animals.

Histopathology was undertaken to examine the morphological changes in the hepatic tissue. In the tissue sections, INH exhibited changes in the normal architecture of the parenchyma cells with swelled hepatocytes and loss of the cell boundaries. Liver histology of the control group (Fig. 6A) and the groups treated with 405 and 540 mg/kg of CRM presented the normal morphology (Fig. 6B and C). Each cell displayed well-defined boundaries, round

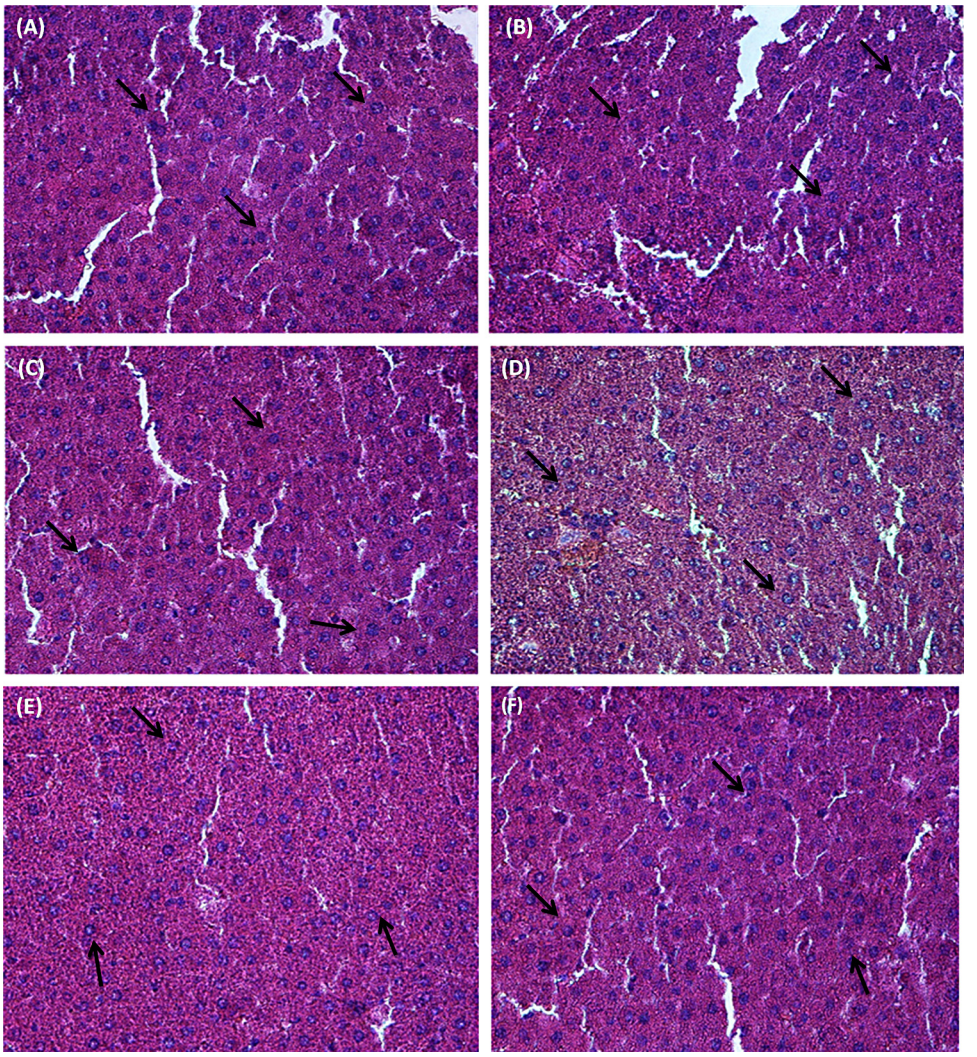

Fig. 7. Histological section of groups treated with INH 150 mg/kg/day (A) and INH 200 mg/kg/day (B); INH 150 + CRM 405 (C); INH 200 + CRM 540 mg/kg (D); conjugate 645 mg/kg/day (E); and conjugate 860 mg/kg/day (F).

vesicular, and centrally located nucleus. Few binucleated cells were also seen in CRM treated groups. Thus, the score was counted as 0 for control as well as CRM-treated animals.

Histological and biochemical data correlated, with an elevation in liver function tests with hepatocellular necrosis in 150 and 200 mg/kg INH-treated groups (Fig. 7A and B). This tissue section showed deeply stained nuclei with patchy liver and was considered as severely damaged in the tissue section of animals administered with high dose. However, the damage was moderate in the animals administered with low dose and hence, histopathology score was taken as "2" and "3," for low and high dose, respectively. Moderate histopathological necrosis was observed in INH + CRM treated groups as displayed in the Fig. 7C and D. Therefore, histopathology score was considered as "2." INH–CRM administration significantly ($p < 0.001$) improved the tissue fabric (Fig. 7E and F) in comparison to INH + CRM. The majority of the hepatic lobules preserved

Table 1
Hepatotoxicity score of control rats, treated with CRM, INH alone, INH co treated with CRM and INH–CRM.

Groups	Histopathology score			
	0	1+	2+	3+
Control	4			
CRM 405 mg/kg	4			
CRM 540 mg/kg	4			
INH 150 mg/kg			1	3
INH 200 mg/kg*				3
INH 150 + CRM 405 mg/kg			2	2
INH 200 + CRM 540 mg/kg			3	1
INH–CRM 645 mg/kg	3	1		
INH–CRM 860 mg/kg	3	1		

0, no injury; 1+, mild injury (a few hepatocytes affected); 2+, moderate injury (necrosis in some hepatocytes); 3+, severe injury (swelled hepatocytes, patchy liver with necrosis). Values are the number of animals with the indicated score. *One animal died during study.

the normal architecture with limited hepatic change and hence histopathology score was expressed as "0." Histopathology score data are compiled in the Table 1. The primary reason for the protective effect of INH–CRM is the antioxidant activity of curcumin due to the quenching of free radical and reactive oxygen species [17].To the best of our knowledge, the conjugation of INH and CRM and study of its effect on INH-induced hepatotoxicity has not been reported in the literature. In the present study, INH–CRM at two doses prevented histological changes associated with isoniazid toxicity. Histopathology results agree with the biochemical findings. Thus, it can be concluded that INH–CRM is efficacious for management of INH-induced hepatotoxicity.

5. Discussion

INH is metabolized by acetylation induced by the hepatic enzyme N-acetyl transferase. INH is acetylated to acetyl isoniazid, which is hydrolyzed into acetyl hydrazine and isonicotinic acid. Polymorphism in NAT-2 has been identified that divides the human population to 'slow' and 'rapid' acetylators. Slow acetylators shunt INH into a secondary metabolic pathway producing hydrazine. Both hydrazine and acetyl hydrazine are toxic metabolites that generate free radicals [3]. This results in reduced levels of glutathione, glutathione-S transferase, catalase and superoxide dismutase. Glutathione represents the non-enzymatic scavengers while glutathione-S transferase, catalase and superoxide dismutase, the enzymatic systems are involved in the detoxification free radicals.

In the present study, INH caused liver injury in rats at the dose levels investigated. The animals treated with the larger INH dose, developed significant hepatic damage with a substantial increase in serum parameter's concentrations. Treatment with INH–CRM resulted in a considerable alleviation of the toxicity, indicated by the alteration of cholesterol, albumin and lipid peroxidation levels. INH–CRM is thus effective in tuberculosis therapy. The effectiveness can be attributed to the antioxidant activity of curcumin.

Hepatic cells are involved in variety of metabolic activities. The transport function of the hepatocytes is particularly disturbed, after liver damage, as a result of deterioration of the plasma membrane. This leads to the loss of functional integrity of cell membranes in the liver [34]. Most of the hepatotoxic chemicals damage the liver, principally by inducing lipid peroxidation directly or indirectly In higher animals, peroxy radicals mediate lipid peroxidation thereby destroying cell membrane integrity, leading to liver injury, atherosclerosis, and kidney damage [35]. In this study work, INH–CRM was effective in reducing the level of malondialdehyde (MDA), a major reactive aldehyde, which appears during the peroxidation of polyunsaturated fatty acid present in the biological membrane [36]. The liver of INH-treated rats exhibits massive liver deterioration. The hepatic MDA level is employed as a biochemical indicator of liver tissue destruction [21]. The biochemical results were supplemented with liver histopathology. The liver of INH-treated group exhibits massive liver deterioration. INH–CRM administration significantly improved the tissue fabric. The histological observation is further supported by serum levels of TG, albumin, cholesterol and MDA. However, studies on the biochemical mechanisms responsible for the hepatoprotective effect of INH–CRM would be necessary

6. Conclusions

INH–CRM was successfully synthesized by employing glyoxylic acid and EDAC as linker and coupling agent, respectively. DMAP was used as the catalyst. The study of the biochemical parameters has demonstrated that the conjugate possesses significant hepatoprotective activity in comparison to the combination of INH and CRM. Levels of TG, cholesterol, and MDA were considerably significantly reduced after INH–CRM treatment, including an appreciable increase in plasma albumin level. Histopathological evaluations further confirm the effectiveness of INH–CRM treatment in reducing liver necrosis. Hence, based on biochemical and histopathological evaluation, it can be concluded that INH–CRM has the potential for optimizing antitubercular therapy.

Conflict of interest statement

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.toxrep.2014.10.001.

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