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Diallyl trisulfide protects liver against the hepatotoxicity induced by isoniazid and rifampin in mice by reducing oxidative stress and activating Kupffer cells

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ABSTRACT

Background&Aim Diallyl trisulfide (DATS) has been verified to ameliorate hepatotoxicity induced by many drugs, but the protective actions in isoniazid (INH) and rifampicin (RFP) have not been reported. We attempted to elucidate the potential effects and mechanisms of DATS against INH&RFP-caused hepatotoxicity.

Methods Male Kunming mice weighing 18-22g were divided into 6 groups. For the hepatic-protective study, co-administrations of DATS (10mg/kg, 20mg/kg, and 40mg/kg bw, respectively) were orally administered two hours before the INH&RFP (100mg/kg, 100mg/kg bw, respectively) treatments. After 11 days treatments, 10 mice in each group were performed for the carbon clearance test, while the other 10 mice were sacrificed for the collection of serum and livers for further measures, including the levels of serum alanine aminotransferase (ALT), aspartate transaminase (AST) and total bilirubin (T.Bili), the liver index, and liver histopathological examination. Malondialdehyde (MDA), glutathione (GSH), the carbon clearance test, the level of interleukin 1-β (IL-1-β) and the immunohistochemistry of F4/80 marked for activated kupffer cell (KC) were measured to investigate potential mechanisms.

Results DATS co-administration significantly inhibited the increase of liver index and elevation of serum ALT, AST and T.Bili levels induced by INH&RFP, as well as improved hepatocellular structure. The further mechanistic studies demonstrated that DATS co-administration counteracted INH&RFP-induced oxidative stress in mice, which was illustrated by the restoration of GSH levels, and the reduction of MDA levels in liver. Furthermore, DATS co-administration reactivated the KCs inhibited by

INH&RFP, which was illustrated by the increase of carbon phagocytosis, the restoration of the number of activated KCs and IL-1-β levels in liver.

Conclusion DATS effectively protected liver against INH&RFP-induced hepatotoxicity, which might be due to its antioxidant effect and enhancement of KCs' activities.

KEY WORDS Diallyl trisulfide; Kupffer cell; Isoniazid; Rifampicin; hepatic protection; Immune mechanism

INTRODUCTION

Tuberculosis (TB), an infectious disease induced by infection of *mycobacteria*, remains a major public health problem and leading cause of morbidity and mortality in the world. It is estimated that about 1/3 of the world's population have been infected with *mycobacterium* TB, and the new infections occur in about 1% of the population every year[1, 2].

Isoniazid (INH) and rifampicin (RFP) are the first-line drugs for the treatment of TB. Unfortunately, these drugs could cause serious adverse effects including drug induced liver injury (DILI)[3]. Importantly, the DILI induced by INF&RFP may lead to the termination of the TB treatment, which contributes to the emergence of drug-resistant TB strains[4]. Therefore, it's an urgent task to find protective drugs against the injury and/or find alternative drugs against TB.

Though the damage mechanism remains unclear, classical studies focusing on the INH metabolism assumed that: After entering body, INH is mainly metabolized into acetyl-INH by N-acetyltransferase (NAT2) in the liver and then acetyl-INH is hydrolyzed into acetyl-hydrazine and isonicotinic acid. This pathway of metabolism is mainly oxidized by CYP2E1 accompanied by the production of many reactive hepatotoxins, such as acetyldiazene, ketienoe, ion and radical[5-7]. Additionally, a number of INH undergoes a secondary metabolic pathway hydrolyzed into hydrazine by amidase[8]. Both acetylhydrazine and hydrazine will generate the oxidative stress and induce the hepatotoxicity. And a few studies have shown that co-administration of RFP to INH could increase the productions of hepatotoxins such as hydrazine due to its positive effect of CYP2E1 activation[9-11]. Moreover, Metushi et al. have demonstrated that INH also could be oxidized into diazohydroxide. INH itself and the

reactive metabolite form- diazohydroxide could covalent bind to the hepatic protein in humans and mice. Both INH-protein and diazohydroxide-protein could induce the increase of INH and an immune hepatotoxicity[12, 13].

Kupffer cells (KCs), resident in the liver, are the largest macrophage population in the liver. The major function of KCs is to phagocytize foreign material, including both opsonized and non-opsonized particles as well as to synthesize and release proinflammatory cytokines, such as TNF-α. Both the macrophage inhibition and increased inflammatory reaction of KCs will induce a liver injury[14]. Though more and more hepatotoxins have been verified to casue damage via KCs[15-17], it's unclear whether INH&RFP could also induce hepatotoxicity by targeting on KCs.

Diallyl trisulfide (DATS) is an organic sulfide that riches in *S-allyl* cysteine extracted from garlic. It has been well demonstrated that the organsulfur compounds are the major component for the beneficial effects of garlic and its related products such as garlic oil[5,11], which has been reported to have series biological effects, including anti-oxidant, anti-tuberculosis, and anti-inflammation[12-14]. Moreover, recent studies showed that DATS could alleviate various hepatotoxic effect by ethanol, naphthalene, carbon tetrachloride (Ccl_4), arsenic et al. through attenuating oxidative stress[18-21]. More interestingly, DATS was also reported to regulate the immune responses and enhance the function of macrophage[20, 22, 23]. These all imply that DATS could be a potential drug to prevent the damage caused by INH&RFP.

To address the above question, our study was aimed to investigate the effects of DATS on INH&RFP-induced liver injury. As known, the co-treatment of INH&RFP is a standard regimen for anti-TB therapy clinically. We use a nonlethal, short-term

mouse model with co-administration of INH&RFP to certify it. The dose and time frame of INH&RFP was defined according to the human-equivalent doses of mouse as well as reference from previous studies . In those reports, the dose scopes of INH were given from 50 to 200mg/kg bw, and the dose scopes of RFP were given from 100 to 200mg/kg bw[24,25,26, 27]. To ensure the hepatic injury and no lethal dose of the co-treatment regimen in mice, a pilot study was performed prior to our formal experiment and we found the dose of 100mg/kg bw+100mg/kg bw was the best regimen for the short term model. And based on this model, we found that DATS effectively protected liver against INH&RFP-induced hepatotoxicity, which might be due to its antioxidant effect and enhancement of KCs' activities.

MATERIALS and METHODS

Meterials

DATS (purity> 97%) was purchased from Chia Tai of Jiangsu CN (Chia Tai, China); Isoniazid Tablets ($C_6H_7N_3O$ 0.1g INH/tablet) and Rifampicin capsules ($C_{43}H_{58}N_4O_{12}$ 0.15g/capsule),which were produced by Xinyi of Shanghai CN (Xinyi, China), were obtained from Qilu Hospital, and then INH and RFP were 1:1 dissolve into physiological saline; Corn oil was purchased from local market of Jinan CN (GB19111, Gold Embryo CORVOIL Co., Shandong, China); Carbon ink was purchased from Chemical Reagent of Beijing CN (Che Rea, China); ALT kit, AST kit, and T.Bili kit were purchased from Biosino of Beijing CN (Biosino, China); GSH assay kit and MDA assay kit were purchased from Njjcbio of Nanjing CN (Njjcbio, China); Mouse IL-1-β sunny ELISA was purchased from Multi Sci of Beijing CN (Multi Sci, China); Rat anti-mouse-F4/80 serotec was purchased from AbD of Oxford UK (AbD, England); Rat IgG immunohistochemistry kit was purchased from Boster of Wuhan CN (Boster, China).

Animals' Treatment

SPF male Kunming mice (18-22g) were provided by the Animal Center of Shandong University (Jinan, China), Certificate of Laboratory Animal: SYXK (Jinan China) 20100011. 120 mice were maintained at approximately 22℃ with a 12-h light: 12-h dark cycle, and had free access to standard chow and tap water. After 5 days acclimation to the laboratory conditions, the animals were randomly divided into 6 groups (n=20): mice in the INH&RFP+DATS groups and DATS group were treated

with DATS (10mg/kg, 20mg/kg, 40mg/kg, and 40mg/kg bw, respectively) by gavage every day, while the mice in the control group and INH&RFP group received equal volume (0.1ml/10g bw) of corn oil. Two hours after the DATS administration, all the animals except those in the control group and DATS (40mg/kg bw) group orally received an INH&RFP (100mg/kg and 100mg/kg bw, respectively), the control group and DATS (40mg/kg bw) group orally got an equal volume (0.2ml/10g bw) of physiological saline. During the treatments, the body weight was measured at 1, 4, 8, 11 days. After 11 days of co-administration, 10 mice of each group were anesthetized at 24 hours after the last treatment. Blood was collected by eyeball extract method and centrifuged at 1500×g for 20 minutes at 4 ℃ to obtain serum. Liver was stripped and weighed. A portion of the liver was fixed in paraformaldehyde (4%) for histopathology and immunohistochemistry, while the other portion of liver tissue was quickly frozen in liquid nitrogen before storing at -80 ℃. The other 10 mice in each group were injected in India ink through tail vein (*i.v.*) to measure the phagocytic capacities by the method of Hudson's *et al*[10].

All animals procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals which were approved by the Animal Experimentation Committee of Shandong Universtity. All efforts were made to minimize animal suffering during experiment.

Measures of Serum Biochemical Index

The levels of serum alanine aminotransferase (ALT) (rate method, YZB0694, Beijing, China), aspartate aminotransferase (AST) (rate method, YZB0693, Beijing, China),

total bilirubin (T.Bili) (diazonium salt method, YZB0121, Beijing, China) were measured by GLAMOUR 1600 random access clinical analyzer (Buenos Airess, Argentina) according to the protocols from manufacturers.

Measures of Mice Phagocytic Capacities----Carbon clearance test

The phagocytosis was measured *in vivo* using carbon clearance method as previously reported[16]. In brief, carbon ink diluted with saline injections (dilution 1:3) was injected to mice (*i.v.,* 0.1ml/10g bw) at the 2 minute, 10 minute interval after ink injection, and then 20μl of blood, taken from the inner canthus venous plexus, were added to 2ml 0.1%Na_2CO_3. After that, the mice were sacrificed, and the liver and spleen were stripped, weighted. The absorbance (OD) of the solutions was measured at 600 nm by Infinite M200 PROV of TECAN (Mannedorf, Switzerland) and phagocytic index (a) was calculated as following formula.

$$K = \frac{\log_{10} OD1 - \log_{10} OD2}{t2 - t1}$$

$$a = \frac{body\ weight}{liver\ weight + spleen\ weight} \times \sqrt[3]{k}$$

H&E Staining

Liver histopathological examination was performed using hematoxylin and eosin (H&E) staining. Slices of 5μm were prepared using paraffin slicer (Thermo), and then

deparaffinized, rehydrated, stained with H&E and then viewed by Olympus AX70 microscope of Tokyo JPN (Olympus, Japan).

Immunohistochemistry of F4/80 marker for KCs

The activation of KCs was measured using immunohistochemistry detection of F4/80 which is the well characterized and extensively referenced mouse macrophage marker[17]. Briefly, Liver paraffin sections (5μm) were deparaffinized, blocked using 3% H_2O_2, and 5% normal goat serum, and then were incubated with rat monoclonal antibody against mouse F4/80 (1:200) (serotec, MCA497G, Oxford, UK) at 4℃ overnight. The following steps were performed strictly according to the procedure of a rat IgG immunohistochemistry kit (SABC method, BA1005, Wuhan, China), and then viewed by Olympus AX70 microscope of Tokyo JPN (Olympus, Japan).

Antioxidant Status Assay in the Liver

liver tissues were homogenized in ice-cold 0.8% saline (w/v= 1:9), and then centrifuged at 1000×g for 20min.at 4℃. The supernatant was collected and stored at -80℃ for antioxidant assay.

The levels of T-GSH, GSH and GSSH in the liver were determined by the 5, 5-dithio-bis-2-nitrobenzoic acid assay using the assay kit (Micro ELISA method, A061-1, Nanjing, CN) according to the method described by the manufactures. The reaction products, which were depended on the amounts of T-GSH and GSSH, had an OD at 405nm by Infinite M200 PROV of TECAN (Mannedorf, Switzerland). And the amount of GSH was obtained by subtracting the two T-GSH, GSSH.

Malondialdehyde (MDA) content was measured by the accumulation of thiobarbituric acid-reactive substance and expressed for the Lipid peroxidation (LPO).Briefly, MDA reacted with 2-thiobarbituric acid (TBA) and a pink-colored product, which was developed depended by the concentration of MDA, had an OD at 532 nm by Infinite M200 PROV of TECAN (Mannedorf, Switzerland). The levels of MDA (nmol/ (mg pro)) were analyzed using commercial assay kits (TAB method, A003-1, Nanjing, CN) according to the manufacturer's instructions

Quantification of IL-1βlevels in liver

The levels of IL-1β in liver were determined using an ELISA kit (EK201B2, Multi SCI, CN) employed the quantitative sandwich enzyme immunoassay technique. Briefly, the sample and standards were added to ELISA plate which had been pre-coated with a monoclonal antibody specific for IL-1β, and IL-1β present was bound by the immobilized antibody. The unbound substances were washed away, and a biotin-linked monoclonal anti body specific for IL-1β was added to the wells. After a wash to remove any unbound substances, streptavidin-HRP was added. After washing, substrate solution was added and the color, which has an OD at 450nm measured by Infinite M200 PROV of TECAN (Mannedorf, Switzerland), developed in proportion to the amount of IL-1β.

Statistical analysis

All data were expressed as mean and standard deviation (SD). SPSS18.0 statistical software was used for statistical analysis. Data was analyzed using one-way ANOVA to compare the means among different groups and Tukey. For the comparisons

between two experimental groups (i.e. INH&RFP group versus control group or INH&RFP+DATS groups versus INH&RFP group) was used LSD Test by SPSS 18.0 to be analyzed. A p value <0.05 was considered significant.

RESULTS

Effects of DATS alone on the liver

The DATS only administered group was added, the results there showed that only with DATS no statistically significant differences in body weight and serum ALT, AST, T.Bili were observed compared with control group. Normal lobular architecture with normal cell morphology and no obvious pathological state were founded in both DATS group and control group. These all intimated that DATS is a safe drug in the study dose to the liver. (Figure 1 & Figure 2& Figure 3& Table 1 &Table 2)

Effects of DATS on INH&RFP-Induced Hepatotoxicity

Effects of DATS and INF&RFP on the body weight, liver weight and liver index

The body weights of mice were consecutively monitored in 11days. After INH&RFP treatment, the body weight of mice showed a negative growth in INH&RFP group. At the end of 11days, the final body weight of INH&RFP group was reduced by 4% of the initial value, while that of control group was increased by 19% (P<0.01). By the DATS co-administration, INH&RFP+DATS groups significantly reversed the body weight depression induced by INH&RFP (5%, 10%, 5% in 10, 20, 40 mg/kg bw, respectively) (P<0.05, P<0.01, P<0.01) at the end of 11days. (Figure1 & Table 1)

Table 1 showed the final body weight and liver weight of mice in six groups. The relative liver weight in INH&RFP group was increased by 84.76% compared to control group (P<0.01). Co-administration of 10, 20 and 40 mg/kg DATS significantly reduced the relative liver weight as compared with INH&RFP group (P < 0.01) by 12.5%, 17.40%, 24,28%, respectively. (Table 2)

DATS co-treatment attenuated INF&RFP-induced increase of serum ALT, AST and T.Bili levels

The levels of serum ALT, AST and T.Bili were elevated in INH&RFP group compared to control group (P < 0.01) (Figure 2): ALT activity increased from 45.98 ±6.79 to 123.5±15.89U/L, p<0.01; AST activity increased from 100.44±14.89 to 191.85±40.89U/L, p<0.05; T.Bili activity increased from 0.38±0.71 to 11.36±2.78, which indicated the hepatic injury. DATS (10mg/kg, 20mg/kg, 40mg/kg, respectively) co-administration groups were ameliorated in the levels of ALT and T.Bili (P<0.05 or P<0.01) (Figure 2A) (Figure 2C), the levels of AST was also significantly refined in INH&RFP+DATS (20 and 40mg/kg, respectively) compared with INH&RFP group (P<0.01 or P<0.05) (Figure 2B)

DATS co-treatment improved the liver histology in INH&RFP-intoxicated mice

The control and DATS group (40mg/kg bw) had normal lobular architecture with normal cell morphology and no obvious pathological state: cells closely packed in a funicular, the cytoplasm with red dye, the nucleus homogeneous with blue dye. The INH&RFP group emerged typical and obvious pathological characteristics in the portal triad region and other liver regions including loose irregular arrangement of

liver cell, a large number of large, round cavity cells appeared and cell necrosis; nuclei were large and deep dye, central venous blood stasis. Compared with INH&RFP group, INH&RFP+DATS (10mg/kg bw) had a small amount of small vacuoles cells. But in the INH&RFP+DATS (20 and 40mg/kg bw) group, the hapatic injury were obviously recovered. (Figure 3)

DATS co-treatment effectively blocked INF&RFP-induced decrease of GSH and increase of MDA

The levels of T-GSH, GSH in INH&RFP had a significant reduction in 31% (P<0.05), 51% (P<0.01) compared with control group. The contents of GSSH were higher than control group up to 103% (P<0.01), but this damage were well reversed by co-administration of INH&RFP and DATS (10, 20 and 40mg/kg bw; respectively). The levels of T-GSH were increased by 17%, 20%, 45% (P<0.05), respectively. The levels of GSH were increased by 48% (P<0.01), 51% (P<0.01), 95% (P<0.01), respectively. And the levels of GSSH were declined by 26% (P<0.05), 38% (P<0.05), 40% (P<0.01), respectively. (Table 2)

The levels of MDA are an index of the intensity of lipid peroxidation damage in the liver. The results showed that INH&RFP group had a higher increase of 164% (P<0.01) than control group, while with the DATS co-administration, the INH&RFP+DATS (10, 20 and 40 mg/kg bw, respectively) groups were significantly decreased the content of MDA by 47% (P<0.05), 56% (P<0.01), 59% (P<0.01) in comparison with INH&RFP group, respectively, (Table 2)

DATS co-treatment improved the capacities of KCs induced by INH&RFP

DATS co-treatment improved the phagocytic capacities by the assay of carbon clearance

The assay of the carbon clearance test in INH&RFP group was decreased by 39% contrast to the control group, and the results showed that DATS (10, 20, 40 mg/kg bw, respectively) co-administration improved the carbon phagocytic capacities by 62%, 67%, 79% (P<0.01) contrast with the INH&RFP group. (Figure 4)

DATS co-treatment led to the activation percent of the kupffer cells

The immune staining for F4/80 of KCs in the liver showed that a reduction population of activated KCs even despaired in INH&RFP group (Fig 5.). With co-administration of DATS, the KCs in INH&RFP+DATS (10mg/kg bw), INH&RFP+DATS (20 mg/kg bw) and INH&RFP+DATS (40 mg/kg bw) group were in varying degrees of activations than INH&RFP group, and the activations were increased with the increasing dose of DATS. (Figure 5)

DATS co-treatment contributed to the normal secretion of IL-1-βby Kupffer cells

The secretions of IL-1β which was mainly secreted by KCs in the liver were decreased in INH&RFP group contrast with control group (P<0.01). By the DATS (10, 20, 40 mg/kg bw, respectively) treatments, the secretions of IL-1-β were similar with the activation of KCs and was increased by 52% (P<0.05), 82% (P<0.01), 113% (P<0.01), respectively. (Figure 6)

DISCUSSION

DATS has been shown to be an effective drug against many drugs and hepatotoxins induced liver injuries, such as ethanol, naphthalene, carbon tetrachloride (Ccl_4)[18-21, 28], especially due to its powerful antioxidant functions. As a compound purified from garlic, DATS had less toxicity and stronger antioxidant capability than other garlic products. The hepatoprotective ability of DATS has received much studies, and it has been verified to provide multiple benefits, e.g. anti-tuberculosis, immunoregulation[21, 22, 29-32]. But the effects of DATS on the INH&RFP-induced liver damage have not been reported. Thus, the effect of DATS against the hepatotoxicity induced by INH&RFP is worth to be studied.

ALT and AST are the most commonly indexes to assess the liver injury in vitro/vivo: Serum ALT level ia a marker for the hepatotoxic effects while AST level is used to measure the liver function.[33] The co-treatment of RFP also can result in a increase of serum T.Bili level[34]. In this study, the results showed that DATS co-treatment significantly attenuated INF&RFP-induced increase of the serum ALT, AST, and T.Bili levels. Besides, the histological examination showed that INF&RFP treatment led to obvious liver injury shown as irregular arrangement of liver cells, vacuolar degeneration, and necrosis, which were also significantly suppressed by DATS co-treatment. These results strongly suggested that DATS effectively abrogated INF/RFP-induced liver injury.

The mechanism of INH&RFP-induced liver injury has been wildly investigated in the past decades, and the roles of GSH has been highlighted[35, 36]. GSH is the largest percent of non-enzymatic antioxidant in the liver, playing an important role in the antioxidant events[37, 38]. As known, oxidative stress results from an imbalance between

oxidants and antioxidants in favor of the oxidants. For the hepatotoxicity of INH&RFP, except for the over-production of oxidative stress, reduced GSH level after INH or hydrazine administration to rats indicates that the decrease of GSH might be also involved in their hepatotoxicity. In the antioxidant activities, the GSH is depleted by free radicals and other oxygen species produced by the hepatotoxins, such as acetyldiazene, ketienoe, ion and radical metabolized by INH&RFP, and oxidized into GSSH[10, 39]. In this study, we found a 31% decrease of GSH and 103% increase of GSSH after the INH&RFP administration for 11days. Parallelly, in consistent with previous studies, it was noticed that INH&RFP exposure also led to significant increase of hepatic MDA levels, which is a biomarker of oxidative stress and lipid peroxidation. The results showed that MDA was increased 164%. However, DATS co-administration significantly elevated the GSH levels by 17%, 20%, 45%, and suppressed the increase of MDA level by 47%, 56%, 59%, which suggested that the restoration of GSH might be a mechanism for the protective effects and the antioxidant ability might be at least partially account for the protection against INH&RFP-induced liver injury.

In addition to the oxidative stress, a number of studies have suggested that immune system was involved in INH&RFP-induced hepatotoxicity[40, 41]. Metushi et al. demonstrated that Cb-b$^{-/-}$, PD1$^{-/-}$ mice (which have impaired immune tolerance) and the Rag$^{-/-}$ mice (which lack of T- and B- cells) were more vulnerable to INH-induced hepatoxicity compared with the wild type C57BL/6 mice, which suggested that INH treatment led to immunosuppression[42]. Several other studies also suggested that TB treatment could result in immune impairment[43, 44]. In view of the KCs are the largest

macrophages, and is the first step in the immune response of the liver, we detected the number of activated KCs by using immunochemistry assay of the KCs markers, F4/80, the results showed that INH&RFP exposure led to the significant decrease of the KCs number; And then the clearance of the carbon which was often been represented for the phagocytic activity of macrophages (KCs) and the secretion of IL-1-b β were also decreased by 39% and 39.8% after INH&RFP treatment. The results that confirmed the inhibited activity of Kupffer cells reduced by INH&RFP were parallel to the reported impairment of the immune system by INH&RFP as we mentioned. Interestingly, DATS co-treatment led to significant increase of the number hepatic Kupffer cells as well as the increase of the phagocytic capacity. These results suggested that Kupffer cells depletion might be involved in INH&RFP-induced hepatoxicity, and DATS could protect against INH&RFP-induced hepatoxicity by activation of Kupffer cells.

In summary, our study demonstrated that DATS could effectively suppress INH&RFP-induced increase of serum ALT, AST, and T.Bili levels and improve the liver morphological changes, which might be associated with the antioxidant capacity and the immunoregulatory capacity. The results of the current study suggested that DATS might be candidate hepatoprotective drug for TB patients receiving INH&RFP.

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DECLARATION OF INTEREST

1 The authors declare no conflict of interest.

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REFERENCES

1. C. M. Stein, Genetic epidemiology of tuberculosis susceptibility: impact of study design, *PLoS pathogens*, 2011, **7**, e1001189.
2. W. H. O. Organization, Tuberculosis).
3. G. S. Gaude, A. Chaudhury and J. Hattiholi, Drug-induced hepatitis and the risk factors for liver injury in pulmonary tuberculosis patients, *Journal of family medicine and primary care*, 2015, **4**, 238-243.
4. A. Tostmann, M. J. Boeree, R. E. Aarnoutse, W. C. de Lange, A. J. van der Ven and R. Dekhuijzen, Antituberculosis drug-induced hepatotoxicity: concise up-to-date review, *Journal of gastroenterology and hepatology*, 2008, **23**, 192-202.
5. T. Sotsuka, Y. Sasaki, S. Hirai, F. Yamagishi and K. Ueno, Association of isoniazid-metabolizing enzyme genotypes and isoniazid-induced hepatotoxicity in tuberculosis patients, *In vivo*, 2011, **25**, 803-812.
6. S. Attri, S. V. Rana, K. Vaiphei, C. P. Sodhi, R. Katyal, R. C. Goel, C. K. Nain and K. Singh, Isoniazid- and rifampicin-induced oxidative hepatic injury--protection by N-acetylcysteine, *Human & experimental toxicology*, 2000, **19**, 517-522.
7. N. P. Santos, S. M. Callegari-Jacques, A. K. Ribeiro Dos Santos, C. A. Silva, A. C. Vallinoto, D. C. Fernandes, D. C. de Carvalho, S. E. Santos and M. H. Hutz, N-acetyl transferase 2 and cytochrome P450 2E1 genes and isoniazid-induced hepatotoxicity in Brazilian patients, *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease*, 2013, **17**, 499-504.
8. S. Tafazoli, M. Mashregi and P. J. O'Brien, Role of hydrazine in isoniazid-induced hepatotoxicity in a hepatocyte inflammation model, *Toxicology and applied pharmacology*, 2008, **229**, 94-101.
9. P. J. Jenner and G. A. Ellard, Isoniazid-related hepatotoxicity: a study of the effect of rifampicin administration on the metabolism of acetylisoniazid in man, *Tubercle*, 1989, **70**, 93-101.
10. N. P. Skakun and V. V. Shman'ko, [Synergistic effect of rifampicin on hepatotoxicity of isoniazid], *Antibiotiki i meditsinskaia biotekhnologiia = Antibiotics and medical biotechnology / Ministerstvo meditsinskoi promyshlennosti SSSR*, 1985, **30**, 185-189.
11. D. S. Askgaard, T. Wilcke and M. Dossing, Hepatotoxicity caused by the combined action of isoniazid and rifampicin, *Thorax*, 1995, **50**, 213-214.
12. I. G. Metushi, T. Nakagawa and J. Uetrecht, Direct oxidation and covalent binding of isoniazid to rodent liver and human hepatic microsomes: humans are more like mice than rats, *Chemical research in toxicology*, 2012, **25**, 2567-2576.
13. J. U. Imir G.Metushi, Isoniaziid-induced liver injury and immune respose in mice, *Journal of immunotoxicology*, 2013, 46.
14. U. A.Boelsterli, *Mechanistic Toxicology*, Taylor & Francis, 11 New Fetter Lane , London EC4P 4EE, 2003.

15. Z. X. Liu, S. Govindarajan and N. Kaplowitz, Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity, *Gastroenterology*, 2004, **127**, 1760-1774.
16. M. Chen and J. Gandolfi, Characterization of the humoral immune response and hepatotoxicity after multiple halothane exposures in guinea pigs, *Drug metabolism reviews*, 1997, **29**, 103-122.
17. J. Neuberger and R. Williams, Immune mechanisms in tienilic acid associated hepatotoxicity, *Gut*, 1989, **30**, 515-519.
18. T. Zeng, C. L. Zhang, Z. P. Zhu, L. H. Yu, X. L. Zhao and K. Q. Xie, Diallyl trisulfide (DATS) effectively attenuated oxidative stress-mediated liver injury and hepatic mitochondrial dysfunction in acute ethanol-exposed mice, *Toxicology*, 2008, **252**, 86-91.
19. T. Fukao, T. Hosono, S. Misawa, T. Seki and T. Ariga, The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2004, **42**, 743-749.
20. F. Zhang, Y. Zhang, K. Wang, X. Zhu, G. Lin, Z. Zhao, S. Li, J. Cai and J. Cao, Diallyl trisulfide inhibits naphthalene-induced oxidative injury and the production of inflammatory responses in A549 cells and mice, *International immunopharmacology*, 2015, **29**, 326-333.
21. N. C. Sumedha and S. Miltonprabu, Diallyl trisulfide ameliorates arsenic-induced hepatotoxicity by abrogation of oxidative stress, inflammation, and apoptosis in rats, *Human & experimental toxicology*, 2015, **34**, 506-525.
22. Z. Shigemi, Y. Furukawa, K. Hosokawa, S. Minami, J. Matsuhiro, S. Nakata, T. Watanabe, H. Kagawa, K. Nakagawa, H. Takeda and M. Fujimuro, Diallyl trisulfide induces apoptosis by suppressing NF-kappaB signaling through destabilization of TRAF6 in primary effusion lymphoma, *International journal of oncology*, 2016, **48**, 293-304.
23. F. M. Hung, H. S. Shang, N. Y. Tang, J. J. Lin, K. W. Lu, J. P. Lin, Y. C. Ko, C. C. Yu, H. L. Wang, J. C. Liao, H. F. Lu and J. G. Chung, Effects of diallyl trisulfide on induction of apoptotic death in murine leukemia WEHI-3 cells in vitro and alterations of the immune responses in normal and leukemic mice in vivo, *Environmental toxicology*, 2015, **30**, 1343-1353.
24. L. Sheng, Y. Xue, X. He, Y. Zhu, H. Li, Y. Wu, R. Dang, M. Tang and P. Jiang, Effects of repeated administration of rifampicin and isoniazid on vitamin D metabolism in mice, *Steroids*, 2015, **104**, 203-207.
25. A. F. Wali, B. Avula, Z. Ali, I. A. Khan, A. Mushtaq, M. U. Rehman, S. Akbar and M. H. Masoodi, Antioxidant, Hepatoprotective Potential and Chemical Profiling of Propolis Ethanolic Extract from Kashmir Himalaya Region Using UHPLC-DAD-QToF-MS, *BioMed research international*, 2015, **2015**, 393462.
26. Y. X. Guo, X. F. Xu, Q. Z. Zhang, C. Li, Y. Deng, P. Jiang, L. Y. He and W. X. Peng, The inhibition of hepatic bile acids transporters Ntcp and Bsep is involved in the pathogenesis of isoniazid/rifampicin-induced hepatotoxicity, *Toxicology mechanisms and methods*, 2015, **25**, 382-387.

27. M. S. Thattakudian Sheik Uduman, R. Sundarapandian, A. Muthumanikkam, G. Kalimuthu, S. A. Parameswari, T. R. Vasanthi Srinivas and G. Karunakaran, Protective effect of methanolic extract of Annona squamosa Linn in isoniazid-rifampicin induced hepatotoxicity in rats, *Pakistan journal of pharmaceutical sciences*, 2011, **24**, 129-134.
28. H. Chen, W. Zhu, J. Feng and S. Li, Protective effect of diallyl trisulfide on liver in rats with sepsis and the mechanism, *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban*, 2012, **32**, 657-662.
29. T. Zeng, C. L. Zhang, X. L. Zhao and K. Q. Xie, The roles of garlic on the lipid parameters: a systematic review of the literature, *Critical reviews in food science and nutrition*, 2013, **53**, 215-230.
30. C. K. Lii, C. W. Tsai and C. C. Wu, Garlic allyl sulfides display differential modulation of rat cytochrome P450 2B1 and the placental form glutathione S-transferase in various organs, *Journal of agricultural and food chemistry*, 2006, **54**, 5191-5196.
31. S. K. Srivastava, X. Hu, H. Xia, H. A. Zaren, M. L. Chatterjee, R. Agarwal and S. V. Singh, Mechanism of differential efficacy of garlic organosulfides in preventing benzo(a)pyrene-induced cancer in mice, *Cancer letters*, 1997, **118**, 61-67.
32. A. Hannan, M. Ikram Ullah, M. Usman, S. Hussain, M. Absar and K. Javed, Anti-mycobacterial activity of garlic (Allium sativum) against multi-drug resistant and non-multi-drug resistant mycobacterium tuberculosis, *Pakistan journal of pharmaceutical sciences*, 2011, **24**, 81-85.
33. H. Turktas, M. Unsal, N. Tulek and O. Oruc, Hepatotoxicity of antituberculosis therapy (rifampicin, isoniazid and pyrazinamide) or viral hepatitis, *Tubercle and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease*, 1994, **75**, 58-60.
34. D. Gendrel, M. Nardou, J. F. Mouba, D. Gahouma, A. Moussavou and J. B. Boguikouma, [Hepatotoxicity of the combination of isoniazid-rifampicin in African children. Role of malnutrition and HB virus], *Archives francaises de pediatrie*, 1989, **46**, 645-648.
35. C. Enriquez-Cortina, M. Almonte-Becerril, D. Clavijo-Cornejo, M. Palestino-Dominguez, O. Bello-Monroy, N. Nuno, A. Lopez, L. Bucio, V. Souza, R. Hernandez-Pando, L. Munoz, M. C. Gutierrez-Ruiz and L. E. Gomez-Quiroz, Hepatocyte growth factor protects against isoniazid/rifampicin-induced oxidative liver damage, *Toxicological sciences : an official journal of the Society of Toxicology*, 2013, **135**, 26-36.
36. A. Chowdhury, A. Santra, K. Bhattacharjee, S. Ghatak, D. R. Saha and G. K. Dhali, Mitochondrial oxidative stress and permeability transition in isoniazid and rifampicin induced liver injury in mice, *Journal of hepatology*, 2006, **45**, 117-126.
37. O. I. Aruoma, Nutrition and health aspects of free radicals and antioxidants, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 1994, **32**, 671-683.
38. Y. Z. Fang, S. Yang and G. Wu, Free radicals, antioxidants, and nutrition, *Nutrition*, 2002, **18**, 872-879.

39. I. G. Metushi, J. Uetrecht and E. Phillips, Mechanism of isoniazid-induced hepatotoxicity: then and now, *British journal of clinical pharmacology*, 2016, DOI: 10.1111/bcp.12885.
40. I. G. Metushi, P. Cai, X. Zhu, T. Nakagawa and J. P. Uetrecht, A fresh look at the mechanism of isoniazid-induced hepatotoxicity, *Clinical pharmacology and therapeutics*, 2011, **89**, 911-914.
41. R. J. Warrington, K. S. Tse, B. A. Gorski, R. Schwenk and A. H. Sehon, Evaluation of isoniazid-associated hepatitis by immunological tests, *Clinical and experimental immunology*, 1978, **32**, 97-104.
42. I. G. Metushi and J. Uetrecht, Isoniazid-induced liver injury and immune response in mice, *J Immunotoxicol*, 2014, **11**, 383-392.
43. S. K. Sharma, A. Balamurugan, P. K. Saha, R. M. Pandey and N. K. Mehra, Evaluation of clinical and immunogenetic risk factors for the development of hepatotoxicity during antituberculosis treatment, *American journal of respiratory and critical care medicine*, 2002, **166**, 916-919.
44. S. Tousif, D. K. Singh, S. Ahmad, P. Moodley, M. Bhattacharyya, L. Van Kaer and G. Das, Isoniazid induces apoptosis of activated CD4+ T cells: implications for post-therapy tuberculosis reactivation and reinfection, *The Journal of biological chemistry*, 2014, **289**, 30190-30195.

Figure legends

Figure1.

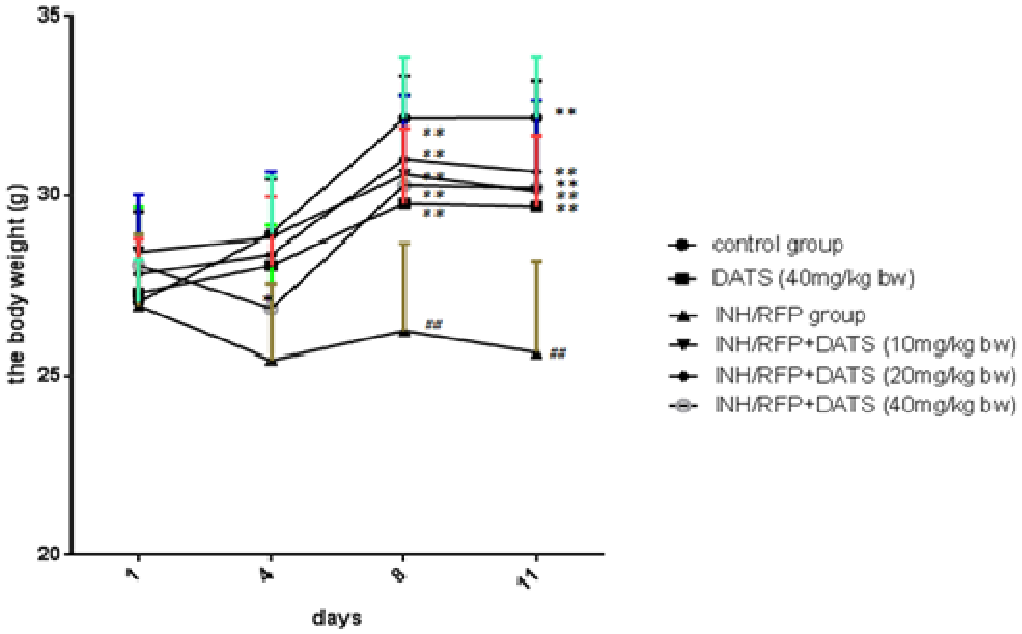
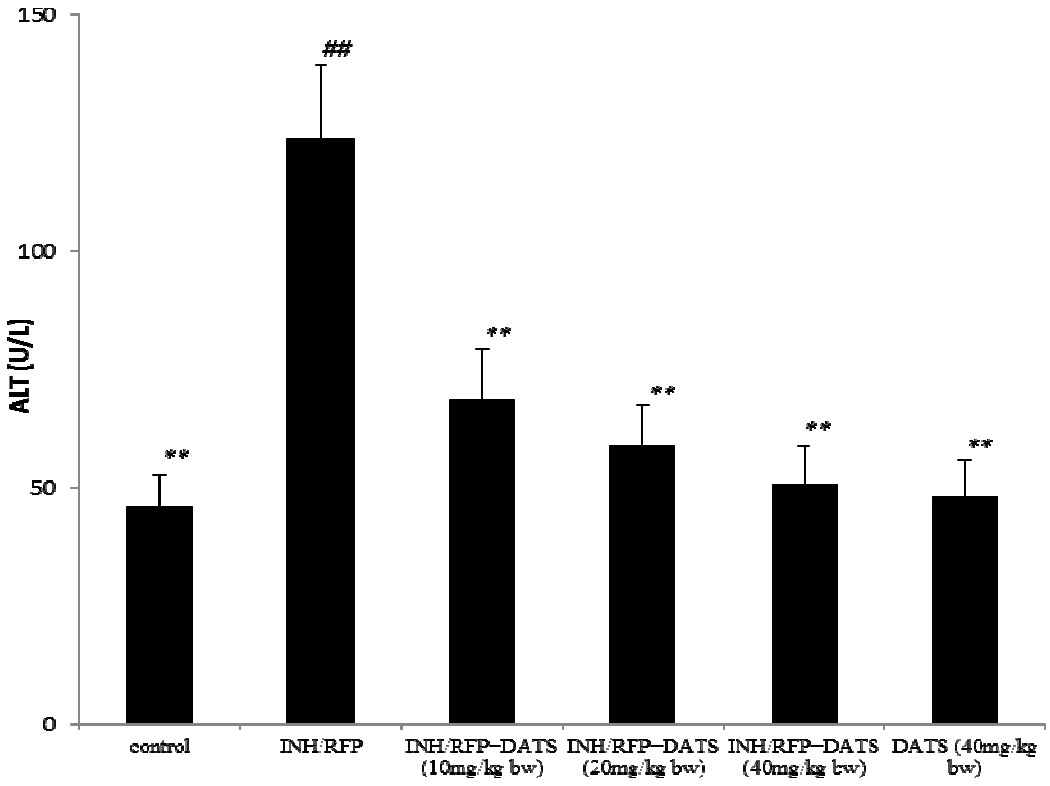


Figure 2.

A)



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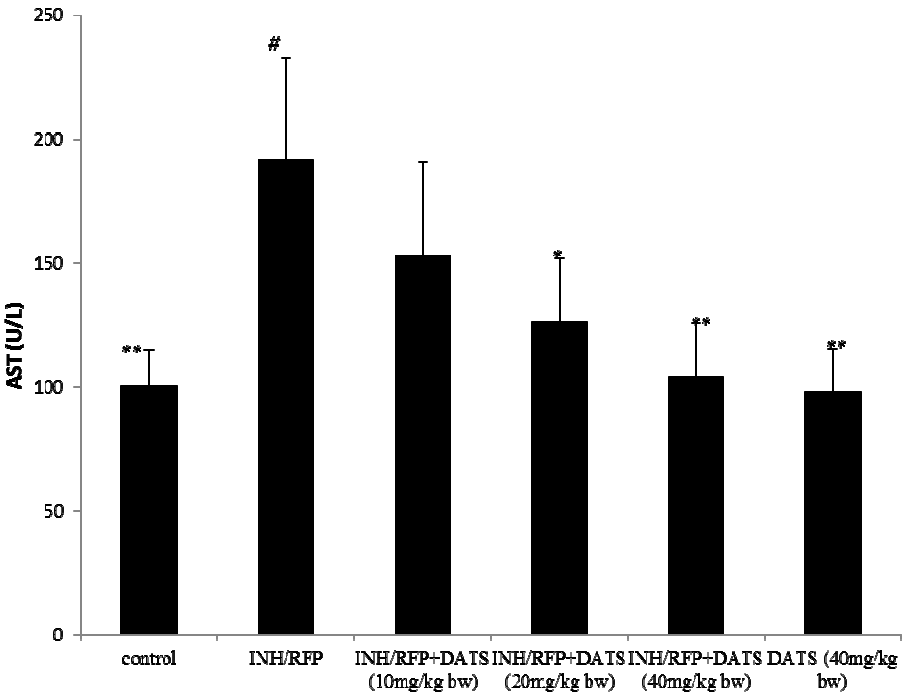

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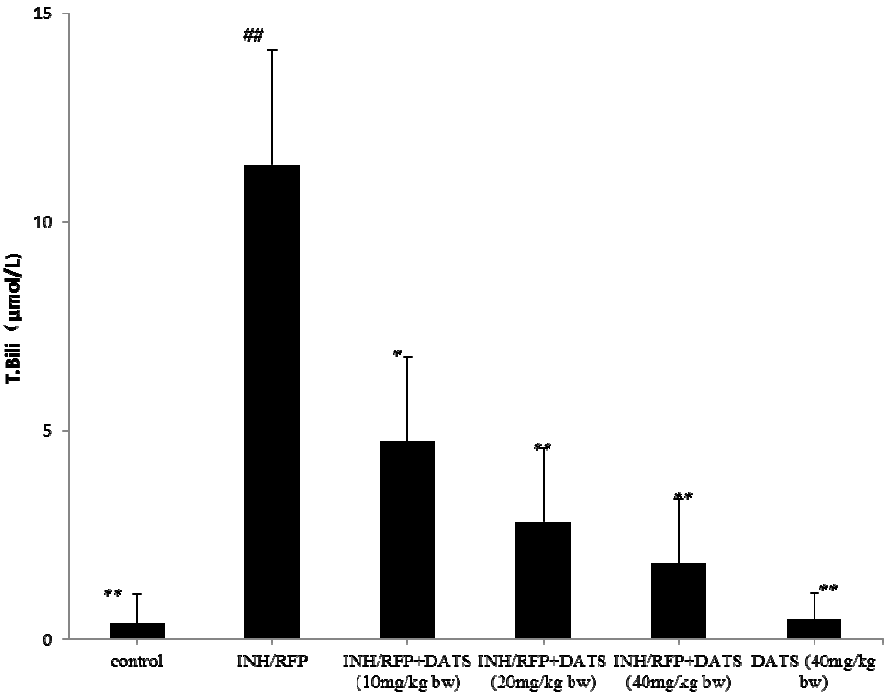

Figure 3.

Seen in the image 1.

A was for control group, B was for DATS (40mg/kg bw) group, C was for INH/RFP group, D was for INH/RFP+DATS (10mg/kg bw), E was for INH/RFP+DATS (20mg/kg bw), F was for INH/RFP+DATS (40mg/kg bw).

Figure 4.

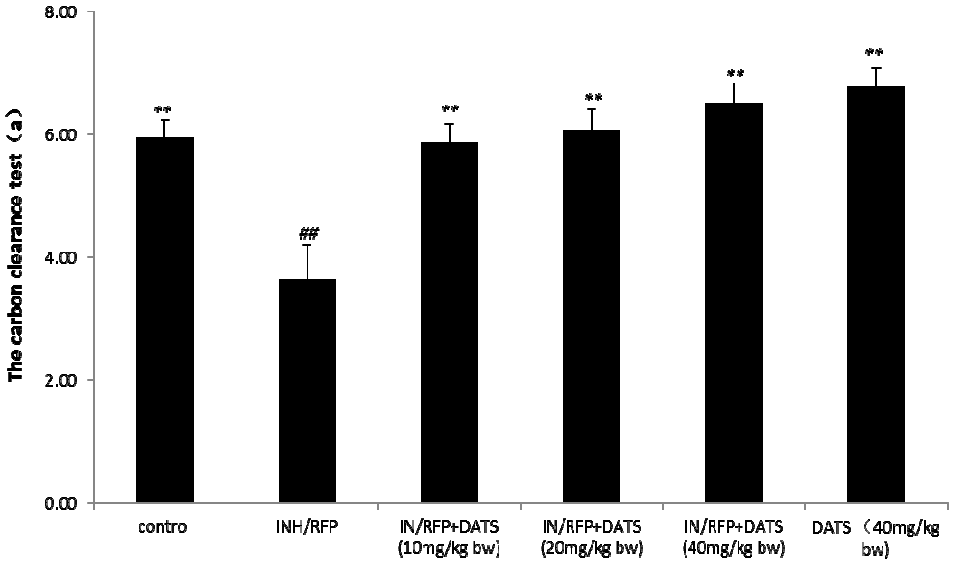

Figure 5.

Seen in the image 2.

A was for control group, B was for DATS (40mg/kg bw) group, C was for INH/RFP group, D was for INH/RFP+DATS (10mg/kg bw), E was for INH/RFP+DATS (20mg/kg bw), F was for INH/RFP+DATS (40mg/kg bw).

Figure 6.

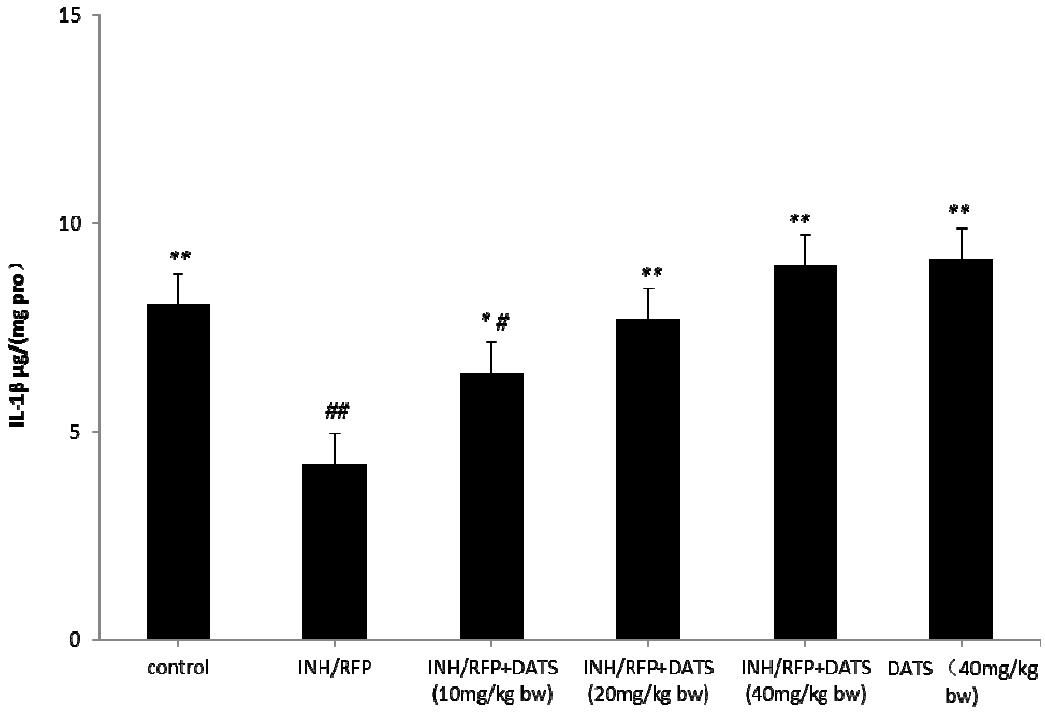

Figure 1. The changes of body weight in six groups during the study shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Figure 2. (A) The levels of serum ALT (U/L) in the six group shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

(B) The levels of serum AST (U/L) in the six group shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$. (C) The levels of serum T.Bili (μ mol/L) in the six group shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Figure 3. The H&E staining of the livers of different groups. Pictures were original captured at 100× magnification. The bar represents 100μm. A was represented for control group. B was for DATS (40mg/kg bw). C was for INH&RFP group. D was for INH&RFP+DATS (10mg/kg bw). E was for INH&RFP+DATS (20mg/kg bw). F was for INH&RFP+DATS (40mg/kg bw)

Figure 4. The assay of carbon clearance test (a) shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Figure 5. The immune stain for F4/80 of KC in six groups. Pictures were original captured at 200× magnification. The bar represents 100μm.

A was represented for control group. B was for DATS (40mg/kg bw). C was for INH&RFP group. D was for INH&RFP+DATS (10mg/kg bw). E was for INH&RFP+DATS (20mg/kg bw). F was for INH&RFP+DATS (40mg/kg bw)

Figure 6. The levels of IL-1- β (pg/(mg pro)) in the six group shown as Mean ± S.D. Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Tables Legends

Table 1. The comparison of body and liver weight in six groups (mean±S.D.)

groups	Final body weight （g）	liver weight （g）	liver index （%）
control group	29.14±2.03**	1.23±0.13**	4.20±0.35**
DATS (40mg/kg)	27.66±2.27**	1.24±0.20**	4.45±0.36**
INH&RFP group	22.75±3.07##	1.82±0.44##	7.76±0.7##
INH&RFP+DATS (10mg/kg bw)	27.13±2.44**	1.84±0.29**	6.79±0.62**#
INH&RFP+DATS (20mg/kg bw)	27.83±2.12**	1.80±0.24**	6.41±0.45**#
INH&RFP+DATS (40mg/kg bw)	27.24±2.04**	1.61±0.22**	5.86±0.40**#

Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Table 2. The levels of T-GSH, GSH, GSSH and MDA (mean±S.D.)

	T-GSH μg/(mg pro)	GSSH μg/(mg pro)	GSH μg/(mg pro)	MDA nmol/(mg pro)
Control group	390.69±30.72**	50.70±14.23**	340.00±29.56**	1.56±0.43**
DATS (40mg/kg bw)	405.98±31.82**	55.80±13.34**	350.18±30.43**	1.58±0.47**
INH&RFP	267.74±57.45$^{\#\#}$	103.01±33.0$^{\#\#}$	164.73±68.62$^{\#\#}$	4.12±0.83$^{\#\#}$
INH&RFP+ DATS (10mg/kg bw)	312.95±47.43*	76.02±26.62*	244.64±50.16$^{**\#\#}$	2.18±0.57*
INH&RFP+ DATS (20mg/kg bw)	320.37±37.36**	63.47±18.67*	249.47±37.10$^{**\#\#}$	1.81±0.46**
INH&RFP+ DATS (40mg/kg	387.42±40.55**	61.80±20.39**	320.62±43.17**	1.69±0.47**

bw)

Compared with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with INH&RFP group, $^{*}P<0.05$, $^{**}P<0.01$.

Abbreviations

DATS diallyl trisulfide

INH isoniazid

RFP rifampicin]

ALT aminotransferase

AST aspartate transaminase

T.Bili total bilirubin

MDA Malondialdehyde

GSH glutathione

IL-1-β interleukin 1-β

KC kupffer cell

T-GSH total-glutathione

GSSH oxidized glutathione

TB Tuberculosis

DILI drug induced liver injury

TBA 2-thiobarbituric acid

SD standard deviation

ROS reactive oxygen species

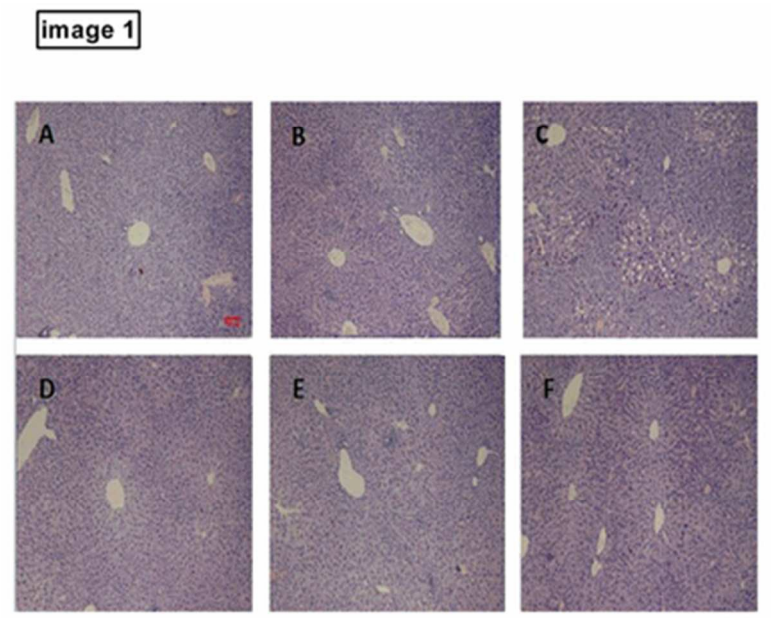

Image 1
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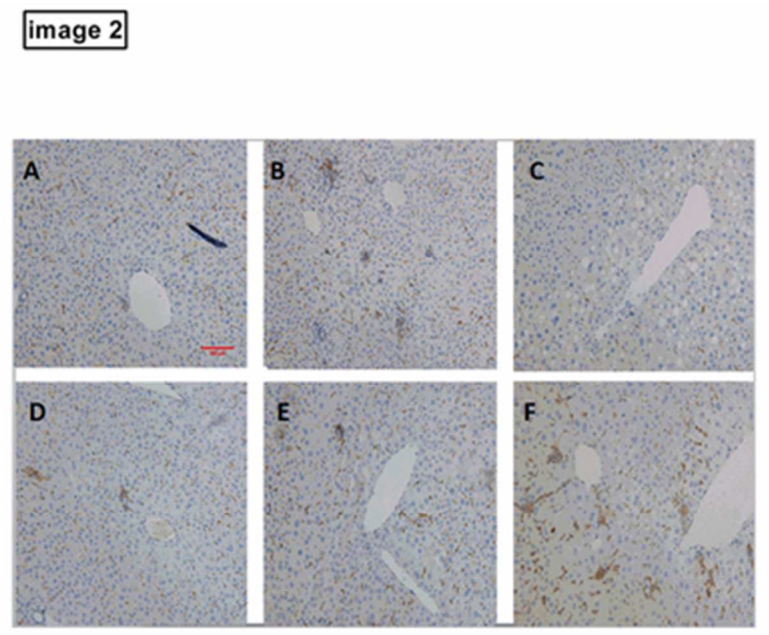

Image 2
16x13mm (600 x 600 DPI)