Protective Effects of Silymarin in Isoniazid Induced Hepatotoxicity in Rabbits

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Background: Isoniazid (INH), first line drug for antitubercular treatment, induces hepatic necrosis in some of human beings in normal doses and in experimental animals in toxic doses. Reactive toxic metabolites of INH are released which disrupt hepatocellular membrane and cause necrosis. So there is a need to find some hepatoprotective and antioxidant agent. Silymarin has been used as a raw extract from the seeds of *Silybum marianum* (carduimariae fructus) but now mixture of 4 flavonolignane isomers is available i.e. silybinin, isosilybinin, silidianin and silychristin. The data suggests that factor of antioxidation is important in the process of hepatoprotection by silymarin. In this study the role of silymarin against INH induced hepatotoxicity was observed in rabbit model.

Methods: Study was conducted on oryctolagus cuniculus rabbits (1-1.5 kg) of either sex for a period of 11 days. Four groups of rabbits were formed at random with 6 animals per group. In group I liver function tests were recorded without any drug. In group II effects of silymarin (50 mg/kg/day p.o) was observed. In Group III isoniazid (50 mg/kg /day i.p) was administered. In group IV combined effects of isoniazid and silymarin were observed. Liver function tests including serum bilirubin, ALT and AST were performed on 12^{th} day. Rabbits were sacrificed on 12^{th} day for observing histopathological changes in liver.

Results: In group I, II and IV there was no mortality and rabbits of group II and IV gained weight. Liver architecture remained almost intact in group II. In group III, bilirubin level increased ($P<0.05$). Histological picture revealed ballooning degeneration, portal inflammation and necrosis of hepatocytes which are signs of acute inflammation. In group IV there was significant improvement in liver architecture when compared with group III. So it can be concluded that INH induced biochemical and histopathological changes are well antagonized by concurrent administration of silymarin for 11days.

Key Words: Hepatotoxicity, INH-induced hepatotoxicity, Silymarin, Liver function tests.

Introduction

During anti TB treatment hepatotoxicity is common side effect and it may range from rise in levels of transaminases up to acute liver failure (ALF).[1] Ten to 20% of patients receiving isoniazid manifest liver injury and some may develop hepatitis. In human beings isoniazid is metabolized at variable rates and this rate is controlled genetically.[2]

Isoniazid (INH, isonicotinic acid hydrazide) is a synthetic antibiotic introduced in 1952 which is bactericidal against replicating Mycobacterium tuberculosis. The drug acts by inhibiting the oxygen-dependent steps in the synthesis of mycolic acid, a component of the mycobacterial cell wall. Administrarion of Isoniazid (INH) has been reported to cause hepatic necrosis in some of human beings in normal doses and in experimental animals in toxic doses. Reactive toxic metabolites of INH are released which disrupt hepatocellular membrane and cause necrosis. So there is a need to find some hepatoprotective and antioxidant agent.[3] INH toxicity can be reduced prophylactically with *pyridoxine*. For treatment it is strongly recommended that drug should be administered concurrently with another agent.

In recent studies it has been observed that when experimental animals are treated with anti-tuberculosis drugs, a correlation exists between hepatic injury and oxidant stress.[4,5-10] As all antitubercular drugs have hepatotoxic effects, experimental studies are in progress to avoid or decrease the toxicity by the use of herbal drugs and/or synthetic compounds. Garlic,[7] silymarin,[5,8] N-acetylcysteine[9,10] and several other herbal drugs prove to have these effects. It is important to note that the inhibition of CYTP-450 2E1 and antioxidant actions show the common mechanism of action of herbal drugs.[5-8,11-13] Among the herbal drugs, silymarin is being used as a dietary supplement for hepatoprotection for over 2000 years. Silymarin, commercially available as Milk Thistle, is an extract from the seeds of *S. Marianum*. Silybines (A and B isomers), isosilybines (A and B), silychristine and silydianine are active flavonoids present in silymarin extract. Silymarin has been found to be safe in animal models, and no significant adverse reactions have been reported in human studies.[14] In this study, we observed the hepatoprotective effects of silymarin in rabbits treated with isoniazid.

Pharmacology and properties of silymarin were observed by Flora et al and described that clinical trials in patients with acute and chronic liver disease prove that silymarin is helpful in improving both acute and chronic viral, drug and alcohol induced hepatitis. Silymarin and its active constituent silybin are reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation.[15]

Pepping[16] found that silymarin can be used as adjunct therapy in patients with a variety of medically confirmed hepatic disorders due to its minimal toxicity and no adverse drug interactions. The data suggested that hepatoprotection by silymarin is due to its antioxidant and antiperoxidative

effects.[17] Due to antioxidant properties of silymarin, INH-induced hepatotoxicity was antagonized by maintaining membrane integrity of hepatocytes in rats.[3]

Materials and Methods

Oryctolagus cuniculus rabbits (1-1.5 kg) of either sex were kept in the animal house of Department of Pharmacology, King Edward Medical University, Lahore. Liberal amount of water and food was supplied. Before starting the experiment all groups were supplied with normal diet and water for acclimatization. Each experiment started with blood collection to record normal values of all rabbits. Number of animals was kept constant in each group i.e. (n = 6). Following Drugs, Chemicals, Kits and Instruments were used.

INH and Legalon (silymarin powder) was obtained from IRZA and LKM Pharmaceuticals Lahore, Pakistan respectively. Following injections, chemicals, kits and equipment and apparatus was used during study. Normal saline 1000 ml I/V soln, lignocain injections vials (local anesthetic), xylene, 10% formaldehyde, alcohol, hemotoxicilin, 1% acid alcohol (HCl), 0.1% ammonia, 1% eosine. Kits for serum bilirubin, ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase). Automated clinical chemical analyzer, automated processing chamber routery microtome, weighing balance (for small animals), electronic weighing balance, UV Spectrophotometer, refrigerator, centrifuge machine, operation table (For small animals), Light microscope, operation light, EDTA containing tubes, disposable syringes 5cc 23G, disposable needles 23G, cotton bags, butterfly needles(22G,23G), scissors, measuring cylinder 100 ml, glass pipette, glass rod, dissection box, filter paper, sharp disposable blades, glass slides, hot plate of 56°C.

Animal Study Protocol

Twenty four rabbits were divided into four groups with six rabbits in each group. In group I normal hepatic function tests were recorded without any drug. In group II effects of silymarin (50 mg/kg /day p.o) were observed. In group III effects of INH (50 mg/kg /day i.p) were observed. In group IV combined effects of INH (50 mg/kg /day i.p) and silymarin (50 mg/kg /day p.o) were observed. Drugs were given once daily for 11 days according to rabbits' weight. Body weights were recorded at start and one day after the last dose.

Blood Sampling

Blood (4 ml) was drawn from marginal ear vein with the help of a fine 22G needle and by putting EDTA containing vial under this for blood collection. Xylene was applied on target surface to dilate vein. Lignocain swab (local anesthetic) was applied on the target to protect from pain. Blood samples obtained from ear vein (before drug treatment) were centrifuged and serum was stored at -20°C till analyzed. A baseline specimens was drawn on 0th day (before administration of any drug) and on 12th day (the day after last day of drug administration).

Biochemical Analysis

Liver function was assessed by levels of bilirubin. Activities of serum ALT and AST before and after the treatment of rabbits with the drugs were measured for hepatocellular membrane integrity. All these parameters were recorded by auto-analyzer using kits.

Histopathological Studies

Small pieces of liver tissues were preserved in 10% formaline and then embedded into paraffin blocks. Thin sections of 5 μM were prepared for microscopic examination. Slides were stained with haematoxylin and eosin (H&E) and observed under light microscope.

Statistical Analysis

The data collected on biochemical parameters was statistically analyzed to test various null hypotheses about the mean values of these parameters. Treatments were estimated at 5% level of significance. Wherever necessary, paired or unpaired 't' tests were also performed.

Results

Effect on Food Intake, Weight of Animals and Mortality

Before drug treatment, baseline liver function tests were performed. In groups I, II and IV water and food intake was normal. In these groups body weights were slightly increased as compared to their weights before drug treatment and no mortality was observed throughout the study. In group III, there was decreased food intake, decreased motor activity and 10% mortality was observed.

Biochemical Analysis

In liver function tests bilirubin, AST and ALT were performed before and after the treatment of rabbits with the drugs on 12^{th} day. In *group I* no drug was administered and mean value of bilirubin, ALT and AST was 0.38 mg/dl, 48.83U/L and 53.83U/L respectively. These values are comparison of 12^{th} day recordings with their baseline values. In *group II* bilirubin level raised, ALT activity decreased and AST activity increased. In *group III* bilirubin level raised ($P<0.05$), ALT activity decreased ($P<0.05$) and AST activity decreased. In *group IV* bilirubin level raised, ALT and AST activities decreased (Table 1).

Comparison of Group III and IV

Serum total bilirubin level of group IV decreased ($P < 0.05$) when compared with group III (Figure 1). Serum AST activity increased ($P < 0.05$) in comparison with group III. (Figure 2).

Histopathological Analysis

In *group I* normal liver parenchyma was observed. Inflammation was observed in portal areas in all drug treated groups. In *group II* fatty changes 0% (n = 0), ballooning degeneration 0% (n = 0), necrosis of hepatocytes 0% (n = 0) and portal inflammation 50% (n = 3). There were

degenerative changes and necrosis in livers of groups III and IV rabbits. However extent of necrosis decreased when silymarin administered. In *group III* fatty changes 0% (n = 0), ballooning degeneration 50% (n = 3), necrosis of hepatocytes 66% (n=4) and portal inflammation 50% (n = 3) were observed (Figure 3). In *group IV* fatty changes 0% (n = 0), ballooning degeneration 16% (n = 1), necrosis of hepatocytes 33% (n=2) and portal inflammation 16% (n = 1) was observed (Table 2).

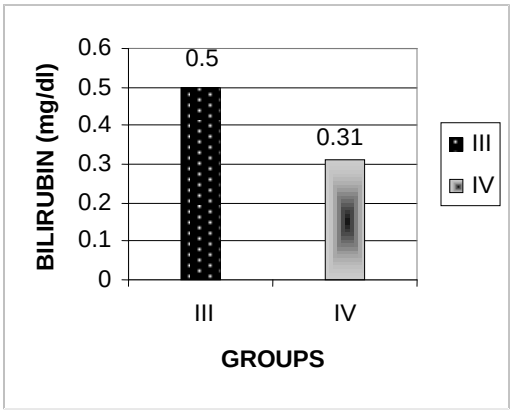

Fig 1: *Comparison of Bilirubin Levels of Group III and IV.*
Group III = INH (50mg/kg /day i.p)
Group IV = INH (50mg/kg /day i.p) + Silymarin (50mg/kg /day p.o)

Table 1: *Effects of different drug treatments on Liver function tests of rabbits.*

Groups		BIL (mg/dl)	ALT (U/L)	AST (U/L)
II	C	0.28 ± 0.04	60.6 ± 8.51	53.8 ± 4.31
	T	0.36 ± 0.06	48 ± 6.91	56.5 ± 3.04
	"p" value	NS	NS	NS
III	C	0.33 ± 0.06	61.6± 9.17	34.3 ± 4.59
	T	0.5 ± 0.03	24.8 ± 2.04	26.6 ± 3.27
	"p" value	*	*	NS
IV	C	0.28 ± 0.04	53 ± 9.69	45.3 ± 8.28
	T	0.31 ± 0.04	43 ± 5.81	44.6 ±6.94
	"p" value	NS	NS	NS

BIL = Bilirubin, ALT = Alanine Aminotransferase, AST = Aspartate aminotransferase, NS = not significant, * = significant (P = < 0.05), C = Control (before drug treatment on 0th day), T = Test (after drug treatment on 12th day)
Group II = Silymarin (50mg/kg /day p.o)
Group III = INH (50mg/kg /day i.p)
Group IV = INH (50mg/kg /day i.p) + Silymarin (50mg/kg /day p.o)

Table 2: *Histopathological Changes (percentage) in Livers in Different Treatments Groups.*

Parameters	Group II	Group III	Group IV
Fatty Changes	0/6 (0%)	0/6 (0%)	0/6 (0%)
Ballooning Degeneration	0/6 (0%)	3/6 (50%)	1/6 (16%)
Necrosis	0/6 (0%)	4/6 (66%)	2/6 (33%)
Portal Inflammation	3/6 (50%)	3/6 (50%)	1/6 (16%)

Digits show number of animals affected out of n=6 rabbits
Group II = Silymarin (50mg/kg /day p.o)
Group III = INH (50mg/kg /day i.p)
Group IV = INH (50mg/kg /day i.p) + Silymarin (50mg/kg /day p.o)

Discussion

Isoniazid (INH), first-line drug in the treatment of tuberculosis, causes mild changes in serum transaminases levels resulting in hepatotoxicity. It is preferred to measure these levels during INH treatment so that the extent of toxicity can be estimated.[2] INH is only 1 to 2% associated with a risk of severe hepatotoxicity. It has been found that hydrazine, INH metabolite, is involved in toxicity.[4]

Hepatotoxin is mainly responsible for increased bile secretion in the serum.[18] Experimental studies on animals suggest that when antitubercular drugs are administered in toxic dose there is rise in ALT, AST and ALP in serum, affecting hepatocellular membrane integrity and its organelles.[19,20] RBC degeneration rate is a measure for binding, conjugating and excretory capacity of hepatocytes. Increased activity of hepatocytes leads to hyperbilirubinaemia which helps to determine integrity of liver.[21]

It has been reported that during sub acute or chronic treatment, INH induced hepatotoxicity occurs in man,[22] rats[23] and guinea pigs[24] resulting in the rise of serum transaminases and phosphatases activities. But in comparison to this there is also evidence that AST, ALT and ALP activities decrease in serum as well as in liver tissues of rabbits after INH administration. Rise in the levels of activities of ALT, AST and ALP in serum followed by fall in these levels in liver tissue indicates drug and chemical induced hepatotoxicity. As extent of INH induced hepatotoxicity is not clearly shown in the serum so measuring above mentioned levels in serum alone don't reflect the actual position of INH induced liver toxicity during sub acute treatment.[25] We decided to perform bilirubin, ALT, AST and liver biopsy in order to verify the disturbed functions and actual necrotic and inflammatory changes in the liver. So in our experiments histological parameters were added to support the biochemical findings. Isoniazid-induced hepatitis is associated with ballooning degeneration and focal hepatocyte

necrosis.[26] Histopathological analysis in rabbits with INH induced hepatotoxicity shows inflammation and necrosis at focal and centrilobular regions.[27] In our study same changes were observed so proving the validity of our animal model.

It was observed that body weights of group II and IV increased with no significant differences. Similar observations were documented in earlier studies.[18,28] There was 10% mortality in group III. However there was no mortality observed in group II and IV. This observation correlated with trials in which there was less incidence of mortality in patients receiving silymarin as compared to placebo ones.[14]

In biochemical analysis tests were performed to assess hepatocellular membrane integrity and liver injury. In *group II* bilirubin level increased, ALT and AST activities decreased and increased respectively (Table 1). In histopathological studies we observed that there were minimum reversible changes and only 50% portal inflammation was observed (Table 2). It has been documented in previous study that in viral hepatitis histo pathological changes obtained with silymarin may show better results than those obtained by the measured liver function test.[29]

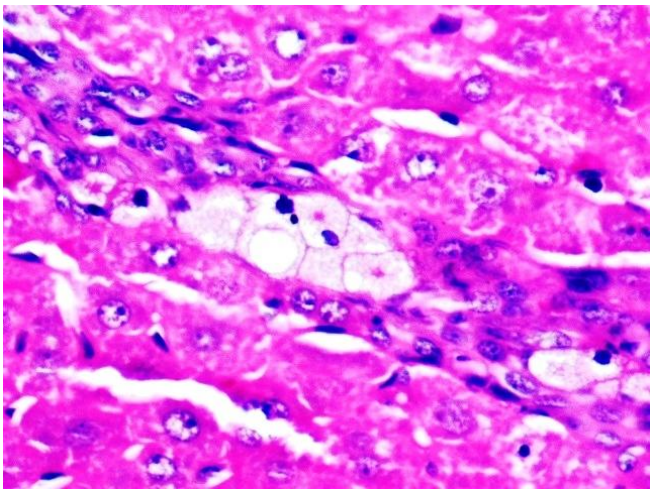

Fig. 3: *Liver Parenchyma of Rabbits (Group III) showing Portal triad inflammation, ballooning degeneration and necrosis (H&E, 40x). Group III = INH (50 mg/ kg/day i.p).*

In group III ALT activity decreased ($P < 0.05$) showing integrity of hepatocytes was abnormal. Serum total bilirubin increased ($P < 0.05$) showing hepatocellular membrane damage (Table 1). Histological picture of liver showed that there was there was 50% ballooning degeneration, 50% portal inflammation and also 66% hepatic necrosis (Table 2, Figure 3).[3] In previous study, histopathological analysis of INH induced hepatotoxicity in rabbits showed inflammation at focal and centrilobular regions.[28] In our study same changes were observed so proving the validity of our animal model.

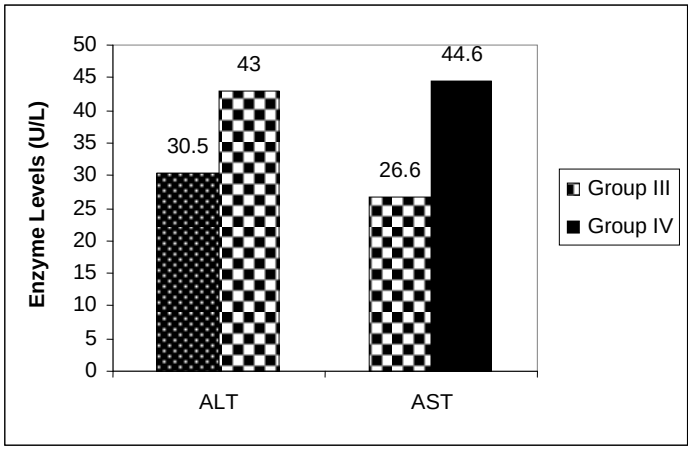

Fig 2: *Comparison of Serum ALT and AST Levels (U/L) of Group III and IV*
Group III = INH (50mg/kg /day i.p)
Group IV = INH (50mg/kg /day i.p) + Silymarin (50 mg/ kg/ day p.o)

In group IV, there was decrease ($P<0.05$) in bilirubin level and AST activity increased ($P<0.05$) when group III and IV were analyzed (Table 1, Figure 1, 2). Silymarin alone don't protect against drug induced toxicity completely. However the combination of INH with silymarin helps to decrease INH induced hepatotoxicity as analyzed from above mentioned biochemical findings. In combined effect (group IV) silymarin decreased the incidence and severity of histopathological changes in liver when compared to group III (Table 2). These findings correlate with the previous studies that silymarin has protective effects against toxicity of AT drugs.[8]

Conclusion

This study showed that silymarin has a significant protection against INH induced hepatotoxicity. Though silymarin is being used as a medicine for more than 2000 years but this is not yet proved to support or refuse its use in patients with liver disease. Good quality, placebo-controlled randomized trials are required, so that silymarin can be introduced as a medicine for use in humans. As there is no significant toxicity in human studies, this plant extract can be administered as a dietary supplement by patients taking anti-tubercular medications.

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Disclosures: None.

References

1. Zaleskis R. The side-effects of TB therapy. *Breathe*., 2005: 2 (1).
2. Mitchell JR, Zimmerman HJ, Ishak KG, Thorgiersson UP, Timbrell JA, Snodgrass WR *et al.* Isoniazid liver injury: clinical spectrum, pathology and probable pathogenesis. *Ann Intern Med.,* 1976.; 84: 181-92.
3. Priya S, Mohan VCR., Pradeep K, Vijayalakshmi P and Karthikeyan S. Membrane stabilizing and antioxidant properties of silymarin against isoniazid induced hepatotoxicity in rats. *Adis Data Information BV.* 2004.
4. Bradford BU, Kono H, Isayama F, Kosyk O, Wheeler MD, Akiyama TE, Bleye L, Krausz KW, Gonzalez FJ, Koop DR and Rusyn I. Cytohcrome P450 CYP2E1, but not adenine dinucleotide phosphate oxidase, is required for-ethanol-induced oxidative DNA damage in rodent liver. *Hepatology.*, 2005; 41: 336–344.
5. Tasduq SA, Peerzada K, Koul S, Bhat R and Johri RK. Biochemical manifestations of anti-tuberculosis drugs induced hepatotoxicity and the effect of silymarin. *Hepatol Res.,* 2005; 31: 132–135.
6. Sodhi CP, Rana SF, Attri S, Mehta S, Yaiphei K and Mehta SK. Oxidative hepatic injury of isoniazid-rifampicin in young rats subjected to protein energy malnutrition. *Drug Chem Toxicol.,* 1998; 21: 305–317.
7. Pal R, Vaiphei K, Sikander A, Singh K and Rana SV. Effect of garlic on isoniazid and rifampicin-induced hepatic injury in rats. *World Gastroenterol.,* 2006; 12: 636–639.
8. Victorrajmohan C, Pradep K and Karthikeyan S. Influence of silymarin administration on hepatic glutathione-conjugating enzyme system in rats treated with antitubercular drugs. *Drugs R D.,* 2005; 6: 395–400.
9. Hogan FS, Krishnegowda NK, Mikhailova M and Kahlenberg MS. Flavonoid, silibinin, inhibits proliferation and promotes cell-cycle arrest of human colon cancer. *J Surg Res.,* 2007; 143: 58–65.
10. Rana SV, Attri S, Vaiphei K, Pal R, Attri A and Singh K. Role of N-acetylcysteine in rifampicin-induced hepatic injury of young rats. *World Gastroenterol.,* 2006; 12: 287–291.
11. Jeong HG. Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. *Toxicol Lett.,* 1999; 105: 215–22.
12. Dai Y and Cederbaum AI. Cytotoxicity of acetaminophen in human cytochrome P450 2E1-transfecred HepG2 cells. *J Pharmacol Exp Ther.,* 1995; 273: 1497–505.
13. Timbrel JA, Mitchell JR, Snodgrass WR and Nelson SD. Isoniazid hepatotoxicity: The relationship between covalent binding and metabolism *in vivo*. *J Pharmacol Exp Ther.,* 1980; 213: 364–369.
14. Attri S, Rana SV, Vaiphei K, Katyal R, Sodhi CP, Kanwar S and Singh K. Protective effect of N-acetylcysteine in isoniazid-induced hepatic injury in growing rats. *Ind J Exp Biol.,* 2001; 39: 436–440.
15. Flora, K., Hahn, M., Rosen, H. and Benner, K. Milk thistle (Silybum marianum) for the therapy of liver disease. *Am J Gastroenterol.,* 1998; 93 (2): 139-43.
16. Pepping J. Milk Thislte: Silybum marianum. *Am J Health Sys Pharma.* 1999; 56: 1195-7.
17. Muzes G, Deak G, Lang I. *et al*. Effect of silimarin (Legalon) therapy on the antioxidant defense mechanism and lipid peroxidation in alcoholic liver disease. *Orv Hetil.,* 1990; 131: 863-866.
18. Rao RR. Mechanism of drug induced hepatotoxicity. *Ind. J. Pharmacol.,* 1973; 5: 313-318.
19. Parthasarathy R, Sarma GR, Janardhanam B, Ramachandran P, Santha T. and Sivasubramanian S. Hepatic toxicity in South Indian patients during treatment of tuberculosis with short-course regimens containing isoniazid, rifampicin and pyrazinamide. *Tubercle.,* 1986; 67: 99-108.
20. Shakun NP and Tabachuk OP. The comparative action of isoniazide, rifampicin and ethambutol on liver function. *Eksp. Klin. Farmakol.,* 1992; 55: 45.
21. Singh B, Saxena AK, Chandan BK, Anand KK, Suri OP, Suri KA and Satti NK. Hepatoprotective activity of verbenalin on experimental liver damage in rodents. *Fitoterapia.,* 1998; 69: 135-140.
22. Mitchell JR, Long MW, Thorgeirsson UP and Jollow DJ. Acetylation rates and monthly liver function tests during one year of isoniazid preventive therapy. *Chest*., 1974; 68: 181-190.
23. Snodgrass W, Potter WZ, Timbrell JA, Jollow DJ and Mitchell JR. Possible mechanism of isoniazid related hepatic injury. *Clin Res.,* 1974; 22: 323.
24. Heisey GB, Hughes HC, Long CM and Rozmiarke H. The guinea pig as a model for isoniazid-induced reactions. *Lab Anim Sci.,* 1980; 30: 42-50.
25. Karthikeyan S. Hepatotoxicity of isoniazid: A study on the activity of marker enzymes of liver toxicity in serum and liver tissue of rabbits. *Indian J Pharmacol.,* 2004; 36 (4): 247-249.
26. Mitchell JR, Zimmerman HJ, Ishak KG, Thorgiersson UP, Timbrell JA, Snodgrass, WR, *et al*. Isoniazid liver injury: clinical spectrum, pathology and probable pathogenesis. *Ann Intern Med.,* 1976; 84: 181-92.
27. Sarich, T.C., Zhou, T., Adams, S.P., Bain, A.I., Wall, R.A and Wright, J.M. A model of isoniazid-induced hepatotoxicity in rabbits. *J Pharmacol Toxicol Methods* 1995; 34: 109-116.
28. Houston S and Fanning A. Current and potential treatment of tuberculosis. *Drugs.,* 1994; 48: 689–706.
29. Dobrilla G and Capurso L. Chapter 14. Epatitie croniche. In: Dobrilla G, Capurso L. Meta-Analisi in Gastroenterologia. Roma: II Pensiereo Scientifico Editore, 2000: 171.