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ORIGINAL ARTICLE

Protective Effect of Different Doses of Zinc Sulfate and Silymarin Combination against Anti-Tuberculosis Drug Induced Toxicity

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ABSTRACT

Objective: To induce hepatotoxicity with Rifampin and INH in albino rats, to estimate histoprotective effect of combination of ZnSO4 and Silymarin.

Methodology: The 'Animal Experimental Study' was carried out of 14 days' duration (starting from day 0-13). A sample size of 28 healthy albino wistar rats were taken and divided into four equal groups (A, B, C and D). Group A was normal control and was given water once a day orally for 14 days. Group B was toxic control and was given INH+ RIF combination (50 and 100 mg/kg/day respectively). Liver protection of combination of ZnSO4 + Silymarin was evaluated at different doses against combination of Isoniazid and Rifampicin induced damage in group C and D. INH+ RIF combination (50 and 100 mg/kg/day respectively) was used to induce liver toxicity in Group B, C and D. Combination effect of regenerative agents (Silymarin + ZnSO4 (100 + 3.5mg /kg/day respectively) and (200 + 7mg /kg/day respectively) was analyzed in Group C + D respectively by measuring liver function profile (ALT, ALK PO4, AST, Total bilirubin).

Results: There was significant elevation of all parameters in Group B, showing liver damage. The level of liver enzymes and total bilirubin was significantly decreased on 13th day (p-value ***< 0.0001) in Group C and D. Comparing Group C, Group D values of only ALT parameter showed significant difference while rest of the parameters did not show any significant difference. A mathematical decrease in AST, ALK PO4, and total bilirubin is evident between Group C and Group D.

Conclusion: The present study has shown the hepatoprotective effect of ZnSO4, SM and their combination in half and full doses as evidenced by improvement in liver function profile (ALT, ALK PO4, AST, Total bilirubin).

Keywords: Albion rats, Hepato-protection, Isoniazid (INH), Liver profile, Liver toxicity Rifampin (RIF), Zinc sulfate

Authors' Contribution:
[1,2]Conception; Literature research; manuscript design and drafting; [2,3]Critical analysis and manuscript review; [5,6]Data analysis; Manuscript Editing.

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Introduction

The liver a vital organ, not only involved in carbohydrates, lipids and proteins metabolism, but also plays a key role in biotransformation and secretion. Liver hepatocytes can detoxify toxic substances.[1] Liver hepatocytes are approximately 80% of liver mass, despite the strong regenerative ability, constant exposure to drugs can cause injury to liver. This is known as drug-induced liver injury (DILI).[2]

Tuberculosis (TB) is serious health problem and one of the leading cause of death, approximately, killing 1.3 million people globally. While anti-tuberculous drugs (ATDs) have made significant progress, but it often leave survivors with post-treatment consequences.[3] Two ATDs isoniazid (INH) and rifampicin (RIF) can cause liver toxicity and cholestasis which can result in the liver enzymes derangements, jaundice and, rarely, acute liver failure. INH intermediate toxic metabolites can cause liver injury. RIF intensifies liver damage by its ability to activate liver enzymes to produce toxic metabolites.[4] ATDs cause inhibition of glutathione and catalase. INH metabolism generates reactive metabolites (acetyl hydrazine and hydrazine). Acetyl groups covalently bind to hepatic proteins and CYP2E1 in a suicidal manner. All these factors contributing to the inhibition of liver regeneration.[5]

Various herbal medicines and nutritional supplements are used to decrease hepatotoxic effects of hepatotoxic drugs.[6] Silymarin (SM) obtained from Silybum marianum, milk thistle has anti-inflammatory and anti-oxidant activities which can support liver regeneration.[7] *Carduus marianum* family plant generates SM, which is used for treatment of various disease. Silymarin hepatoprotection potential studies in rodents have shown that SM treatment reduces biochemical and transaminase levels. SM has anti-oxidant, anti-fibrotic, anti-inflammatory and immunomodulating effects. Its anti-oxidant activity is by reducing free radicals and increasing glutathione.[8]

All body tissues contain zinc. Zinc is the most common intracellular trace element.[9] Zinc, functions as cofactor in more than 240 enzymes and aids in metabolization of carbohydrates and proteins.[10] Zinc micronutrient has a pivotal role in liver metabolism, its deficiency can cause liver damage or even cirrhosis.[11] Trace element Zinc supplementation ameliorate the liver tissue damage.[12] Zinc sulfate improves antioxidant activity. It also has beneficial effects on hepato-renal damage after ischemia or reperfusion.[13]

Keeping all these factors in view we planned to evaluate and compare the hepatoprotective effect of combination of ZnSO4, Silymarin against INH and RIF (ATDs) induced hepatotoxicity.

Methodology

It was an experimental study on animal model and was done in University of Veterinary and Animal Sciences Lahore. The duration of study was 9 months and technique for sampling used was **s**imple random sampling by lottery method. In total, 28 albino male rats (7 rat in one group) were used in this study. The sample size was calculated by using 1% level of significance (α), 95% power of test (β) with expected mean value Silymarin and ZnSO4 for ALT in half doses as 56.7±2.9 and full doses as 63.3±2.9. (14)

$$N= \frac{(\sigma 1^2+\sigma 2^2)\times(Z_1-\alpha/2+Z_1-\beta)^2}{(\mu_0 - \mu\alpha)^2}$$

σ^2 = Variance

$Z_{1-\beta}$ = power of test 95% = 1.65

$Z_{1-\alpha}$ = confidence level 99% = 2.575

μ_0 = population mean 1 = 63.3

$\mu\alpha$ = population mean 2 = 56.7

Sample size for one group = n = 7

Inclusion criteria: Albino male rats weighing 150-200g were included.

Chemicals:

- Isoniazid and Rifampicin were taken from Shazoo laboratories.
- Silymarin was purchased from Jiangxi Medicine through importer.
- Zinc sulfate was purchased from local market.
- SGPT (ALT) liquid UV kit (Chema Diagnostics)
- SGOT (AST) liquid UV kit (Human)
- ALK liquid UV kit (Chema Diagnostics)
- Bilirubin total FL kit (Chema Diagnostics)

Equipment

- Sartorius weighing balance Sartorius
- Chemistry Analyzer: Make-Itly; Model-Sphera

- Centrifuge machine: Make-China capable of 4000 rpm for vacutainers/test tubes
- Incubator (Memmert): Make –Germany; Model: E-07086

In total twenty-eight albino rats of 150-200 g were grouped into four groups (A, B, C and D) under natural day and night cycles at 23±2 º C. Animals were marked for identification and were provided water and libitum and rodent chow. Drugs solutions were prepared in distilled water and administered orally.

Group A (Normal Control): was administered orally 0.3 ml distilled water (DI) for 14 days.

Liver toxicity was induced by Isoniazid (INH) + Rifampicin (RIF) - 50 + 100 mg/kg/day respectively[15] daily once in morning for 14 days in **Group B (Positive Control), C and D.**

Liver-protection was given by (30 min before inducing agent) by combination of (Silymarin + ZnSO4) in **Group C** (100mg/kg/day + 3.5mg/kg/day) and (200mg/kg/day (17) + 7mg /kg/day (16)) in **Group D** for 14 days on daily bases.

Rats were weighed to adjust the dose weekly. One ml blood was drawn via the cardiac puncture[18] using 5cc syringes on 0, 6 and 13th day under ether anesthesia and was allowed to clot and centrifuged at 3000rpm for 10 min to obtain serum (stored at -20 ºC) for biochemical tests.[19] Chemistry Analyzer Sphera was used to perform liver function tests via using commercially available kits. Manufacturer methodology was used. SPSS version 20 and Graph pad prism version 5 were used to enter data. For data analysis one-way analysis of variance + turkey multiple comparison test was used. P-value ≤ 0.05 was significant.

Ethical approval for the study was obtained from the Ethical & Research Committee, King Edward Medical University Lahore (18/ PEC/RC/KEMU) on 31-07-2017.

Table I: Comparison of mean bilirubin and liver enzymes in study groups

Parameter/Days	Group				P-Value
AST	**A**	**B**	**C**	**D**	
0 Day	133.4±17.34	113.6±21.88	132.7±10.67	130.6±13.02	0.0994
6th Day	125.1±10.11	358.3±67.56	185.9±38.84	160.4±17.75	***< 0.0001
13th Day	125.4±9.624	728.3±37.52	200.3±13.76	170.1±15.16	***< 0.0001
ALT					
0 Day	37.29±7.251	37.86±8.611	39.86±3.288	43.00±2.708	0.3014
6th Day	38.00±5.477	78.14±5.460	56.71±1.799	49.71±1.890	***< 0.0001
13th Day	39.00±4.830	104.3±4.071	59.29±2.138	50.43±3.309	***< 0.0001
ALK PO4					
0 Day	117.0±23.59	157.9±42.81	118.7±38.31	164.7±50.90	0.0638
6th Day	143.7±32.77	885.7±131.5	266.1±74.84	201.4±39.92	***< 0.0001
13th Day	145.0±32.24	1210±108.9	333.7±26.34	268.3±31.14	***< 0.0001
Bilirubin					
0 Day	0.01857±0.04914	0.01429±0.03780	0.01429±0.03780	0.01429±0.03780	0.9963
6th Day	0.06143±0.05843	1.214±0.4337	0.6571±0.1813	0.5143±0.2410	***< 0.0001
13th Day	0.04286±0.05345	0.5286±0.1704	0.1714±0.1380	0.1429±0.07868	***< 0.0001

Results

AST in Group A was 133.4±17.34, 125.1±10.11 and 125.4±9.624 U/L at 0, 6 and 13th day. AST in Group B was elevated from 113.6±21.88 to 358.3±67.56 up to 728.3±37.52U/L respectively at 6th and 13th day. AST levels in Group C were elevated from 132.7±10.67 to 185.9±38.84 and 200.3±13.76 at 6th and 13th day. AST levels in Group D were changed from 130.6±13.02 to 160.4±17.75 and 170.1±15.16 U/L at 6th and 13th day. A significant difference was present among groups at day 6th and 13th day with p-value of 0.0994, ***< 0.0001 and ***< 0.0001***p-value ≤ 0.0001, ** p-value ≤ 0.001, * p-value ≤ 0.05

ALT of Group A was 37.29±7.251, 38.00±5.477 and 39.00±4.830U/L at 0, 6 and 13th day respectively. ALT of Group B raised from 37.86±8.611to 78.14±5.460 and 104.3±4.071U/L at 6th and 13th day respectively. ALT of Group C increased from 39.86±3.288 to 56.71±1.799 at 6th day and 59.29±2.138U/L and 13th day. ALT of Group D raised from 43.00±2.708 to 49.71±1.890 at 6th and 50.43±3.309 U/L at 13th day. A significant difference was seen among Group B, C and D at 6th and 13th day with p-value of 0.3014, ***< 0.0001 and ***< 0.0001 respectively.

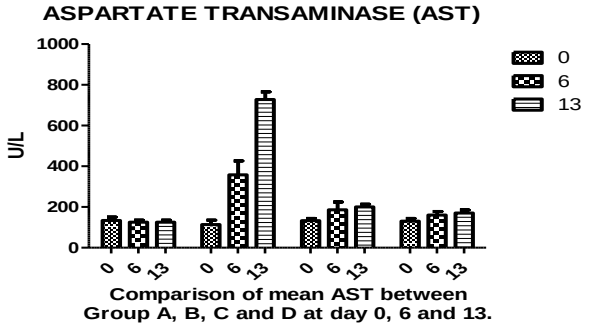
Alkaline Phosphatase in Group A was 117.0±23.59, 143.7±32.77 and 145.0±32.24U/L at 0, 6 and 13 days. ALK PO4 in Group B at o day is 157.9±42.81, at 6th day is 885.7±131.5 and at 13th day is 1210±108.9 U/L. ALK PO4 in Group C is 118.7±38.31, 266.1±74.84 and 333.7±26.34 U/L at 0, 6 and 13 days. ALK PO4 of Group D elevated from 164.7±50.90 to 201.4±39.92 and 268.3±31.14 U/L respectively at day 6th and 13th. Significant difference was seen among the groups on day 6 and 13 with p-value of 0.0638, ***< 0.0001 and ***< 0.0001 respectively.

Total Bilirubin in group A was 0.01857±0.04914, 0.06143±0.05843 and 0.04286±0.05345 mg/dl at day 0, 6 and 13. Total Bilirubin in Group B increased from 0.01429± 0.03780 to 1.214± 0.4337 and 0.5286± 0.1704 mg/dl at day 6 and 13. Total Bilirubin levels of Group C also increased from0.01429±0.03780 to 0.6571±0.1813 and 0.1714± 0.1380 mg/dl at day 6 and 13 respectively. Total Bilirubin of Group D elevated from 0.01429±0.03780 to 0.5143±0.2410 and 0.1429± 0.07868 mg/dl on day 6 and 13 respectively. Significant difference was seen among the groups at day 6 and 13 with p-value of 0.9963, ***< 0.0001 and ***< 0.0001 respectively.

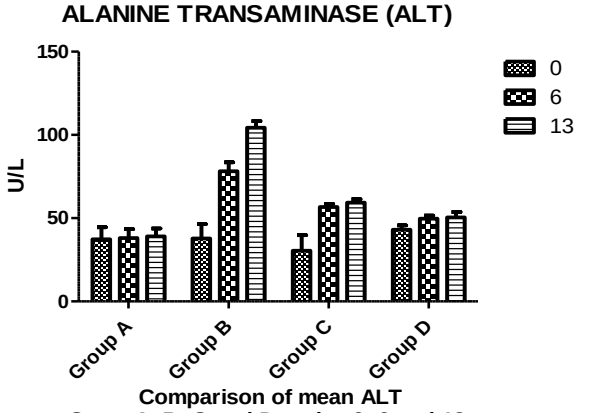
Table II: The Post hoc Tukey's test for comparing LFTs

Group	ALT	AST	ALK PO4	Total Bilirubin
A Vs B	***	***	***	NS
A Vs C	***	***	***	NS
A Vs D	***	**	**	NS
B Vs C	***	***	***	NS
B Vs D	***	***	***	NS
C Vs D	***	NS	NS	NS

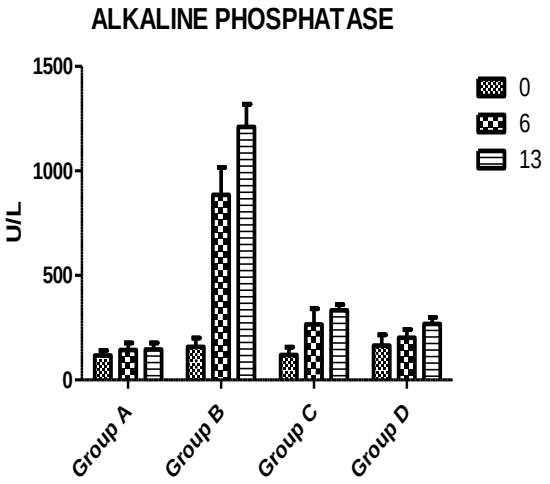
Significant (***p-value ≤ 0.001, ** p-value ≤ 0.01, * p-value < 0.05), Non-significant (NS)



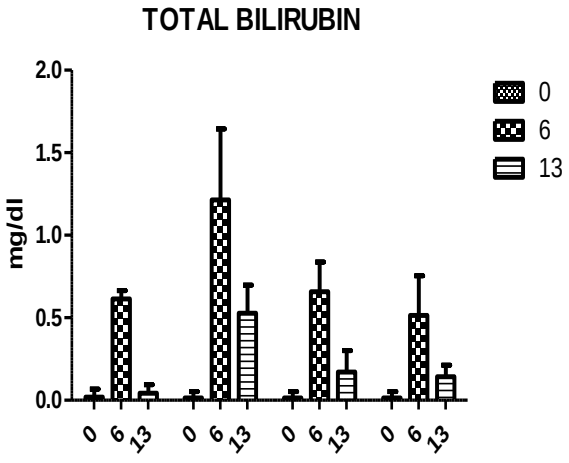
Comparison of mean AST between Group A, B, C and D at day 0, 6 and 13.



Comparison of mean ALT GroupA, B, C and D at day 0, 6 and 13.



Comparison of mean Alkaline Phosphatase GroupA, B, C and D at day 0, 6 and 13.



Comparison of mean Total Bilirubin between Group A, B, C and D at day 0, 6 and 13.

Discussion

Effect of Silymarin + ZnSO4 combination in half (100 + 3.5mg/kg/day) and full (200+ 7mg/kg/day) doses were studied in C and D groups respectively against INH+ RIF induced hepatotoxicity. The serum biomarkers for the detection of liver injury are total bilirubin, ALT, ALK PO4, and AST.[20] ALT is found in the liver cells cytoplasm, which releases into the bloodstream in case of liver injury, hence raised ALT levels indicate liver cell damage. AST, another potential biomarker most found in the heart, but AST is also used as a biomarker as representative of liver injury.[21]

Serum ALT level was significant in B group with ***p-value ≤ 0.001. ALT parameter was reduced in C and D groups as compared to B ***p-value ≤ 0.001. D group showed marked reduction (***p-value ≤ 0.001) in ALT levels as compared to C group. Serum AST level was significant in Group B with ***p-value ≤ 0.001 as compared to Group A. AST levels were reduced in Group C and Group D as compared to Group B ***p-value ≤ 0.001. No significant difference was observed in levels of AST in Group C and Group D. Serum Alkaline Phosphatase level was significant in Group B with ***p-value ≤ 0.001 as compared to Group A. ALK PO4 levels were reduced in Group C and Group D as compared to Group B ***p-value ≤ 0.001. No significant difference was observed in levels of Alkaline Phosphatase in Group C and Group D. Bilirubin, a less sensitive marker only increased when liver damage is very marked.[22] Treatment with INH and RIF in group B caused significant increase in level of total bilirubin as compared to Group A with ***p-value ≤ 0.001. C and D groups as compared to B shows significant improvement in total bilirubin.

Raised liver enzymes are biomarkers of Isoniazid and Rifampicin induced damage possible due to cellular leakage from liver due to generation of reactive oxygen species, toxic metabolites, oxidative damage, mitochondrial toxicity and impair oxidant- antioxidant balance leading to lipids membrane damage or covalently bind with cellular components resulting in hepatocytes damage. The synergistic toxic potential of Isoniazid and Rifampicin is associated with enzyme induction.[23]

Silymarin, exhibits potent liver protective activity via scavenging free radicals, reducing oxidative stress, modulating inflammation by inhibiting NF-κB activation and cytokine production, thus decreasing liver inflammation. Additionally, it increases liver

regeneration by stimulating DNA and RNA synthesis, hepatocyte proliferation and inhibiting fibrogenesis.[24] Results are inconsistent with Elhassaneen, Y.A study, where Silymarin caused a significant decrease in liver enzymes (AST, ALT, and ALK PO4) in comparison with normal control.[25]

Zinc, an essential trace element, has antioxidant, anti-apoptotic and anti-inflammatory activity. It is highly important for cell division, differentiation and signaling. It plays a pivotal role in normal liver functioning. Decreased levels have been estimated in both acute and chronic hepatic diseases. Majority of the liver diseases are attributed to Zn deficiency.[26] It also regulates various body functions. It mainly provides hepatic protection and prevents steatosis which is attributed to its antioxidant effects.[27] Liver enzymes are thought to be a more accurate and sensitive sign of liver damage when they are present in serum and are typically linked to hepatocellular damage. According to Ochuko, O., Obidike, N.A. (2023), a raised ALT, AST, ALK PO4 and bilirubin can be decreased through ZnSO4 supplementation.[28] ALT levels are significantly reduced in C and D groups, when compared with B group. A significant improvement for ALT was observed among group C and D. This study shown that ZnSO4 and Silymarin combination significantly reduced ALT, AST, ALK PO4 and total bilirubin compared to the toxic group. A mathematical decrease in AST, ALK PO4, Total bilirubin, was also evident between Group C and Group D but statistically results are not significant, a prolong study is needed to access the protective role in Group D.

Conclusion

Combination effects of regenerative agents Silymarin and Zinc Sulfate have significant protective effects against anti-TB liver toxicity. Comparing (C Group= Silymarin+ ZnSO4 100 +3.5mg /kg/day respectively) and (D Group = Silymarin + ZnSO4 200 +7 mg /kg/day respectively) D Group have significant improvement in deranged ALT levels.

A mathematical decrease in AST, ALK PO4, and total bilirubin is evident between Group C and Group D. longer duration of study can be done to obtain significant results. This study can also be applied on humans and supplements of zinc and silymarin can be given to TB patients for liver protection.

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