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ESTIMATION OF LIVER ENZYMES AND HISTOPATHOLOGICAL STUDY OF HEPATIC TISSUES IN RABBIT MODELS

MUSHARRAF A[1], HAYAT Z[1], TANZEEM M[1], TARIQ R[2], MASOOD M[2] AND WAQAS M[1*]

1: School of Pharmaceutical Sciences (SPS), Johar Institute of Professional Studies, Lahore, Pakistan

2: School of Medical Sciences (SMS), Johar Institute of Professional Studies, Lahore, Pakistan

***Corresponding Author: E Mail:** waqaskhanjips@gmail.com **+923213535005**

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ABSTRACT

Tuberculosis is a pretty dangerous disease if not pick up accurately, triggered by a gram negative rod *Mycobacterium tuberculosis.* First line anti tuberculosis drugs, considered as top therapeutic approach to eradicate the infectious organism, which are also responsible for causing severe hepatotoxic effects. Silymarin is used since decades to compensate the noxious effects instigated by first line anti tuberculosis drugs. In this study Thymoquinone used as hepatoprotective agent in rabbit models. Our study suggests that thymoquinone appear to produce more reliable hepatoprotective effect as compare to silymarin. Results are supported by histopathological studies and Biochemical analysis. Thymoquinone in concentration of 40mg/kg showed mean value 17.08*** for ALT and 50.376* for AST which is quite better as compare to silymarin.

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, Thymoquinone, Silymarin, AST, ALT

INTRODUCTION

The gram negative rod *Mycobacterium tuberculosis* is the significant contributing agent to root tuberculosis (TB), which is one of the foremost reason of human Death globally. **[1]**. It is transported through airborne droplets **[2]**. When a person encountered with TB infection then the suggested treatment or first line chemotherapeutic agents against the noxious bacteria, comprises of rifampicin (RIF), isoniazid (INH), ethambutol and pyrazinamide **[3, 4]**. CYP450 system is responsible for the metabolism of rifampicin in the liver and intestine **[5]**. RIF may also reported to be responsible for hepatocellular dysfunction **[6]**. The mechanism of RIF hepatotoxicity is still unidentified. There is also not an apparent proof for the occurrence of lethal metabolite. The metabolism of INH is unusual as compared to the other drugs as it is a pro-drug and metabolism is through the acetylation process by the hepatic enzyme N-acetyl-transferase and then hydrolyzed into hydrazine or further acetylated into its metabolite named as di-acetyl-hydrazine. It is reported in previous works that acetyl-hydrazine is a noxious metabolite of INH **[7]**. In combination INH and RIF increase the chances of hepatotoxicity because RIF stimulates the enzyme isoniazid hydrolase which is responsible for the metabolism of INH and increases the hydrazine production especially in individuals which are slow acetylators. Moreover, RIF interacts with numerous antiretroviral drugs through modulation in their metabolic enzymes and influences the level of these drugs in plasma and increases the hepatotoxicity potential **[8]**. Untoward effects that are encountered with the use of INH includes jaundice, hepatitis, peripheral neuritis, high liver transaminases, vasculitis, arthritic syndrome, skin eruption, hematological problems like agranulocytosis; thrombocytopenia, anemia, hypersensitivity like fever, skin rashes. RIF causes hypersensitivity reactions, hepatitis and hematological disorders including hemolytic anaemia, thrombocytopenia and transient leukopenia, fever, rash, nausea, chills, vomiting, nephritis, myalgia, tubular necrosis and shock. High level of serum alkaline phosphatase is also observed **[9]**. Silymarin is usually used to counter the unwanted effects of first line anti TB drugs **[10]**.

Nigella sativa (*N. Sativa*) is commonly known as black cumin or black seed. The *N. Sativa* is a flowering, dicotyledonous plant and belongs to family *Ranunculaceae*. It is grown in Middle East Asia, Central Europe and Western Asia **[11, 12]**. Conventionally,

the seeds of this plant are used as spices, additives and as food preservatives in various societies. Furthermore, seeds and its oil are used for the treatment and prevention of many ailments for more than 2000 years **[13]**. The main active ingredient Thymoquinone (TQ) is isolated from the volatile oil of *N. Sativa* **[14]**.

TQ chemically known as 2-isopropyl, 5-methyl, 1, 4-benzoquinone ($C_{10}H_{12}O_2$) having molecular weight of 164.2g/mol **[15]**. *N. sativa* seeds contain 30-48% of TQ **[16]**. The therapeutic use of *N. sativa* and its active element TQ is due to its anti-hypertensive, anti-inflammatory, anti-histaminic, anti-cancer, immunity modulating and hypoglycemic effects **[17, 18]**.

TQ proposed as its shielding effects against hepato-toxicity connected with the use of anti-tuberculosis. In this research work the hepatoprotective potential of TQ was evaluated in the hepatotoxicity induced animal model by the use of Isoniazid and Rifampicin.

METHODOLOGY

A cross sectional comparative study was conducted at pharmacology lab of Johar Institute of Professional studies (JIPS), Lahore, for a period of 6 months to evaluate the hepatoprotective effect of Thymoquinone. The study was approved by ethical committee of JIPS.

Drugs and Chemicals: RIF and INH having 99% purity were obtained from Pacific Pharmaceuticals Ltd., Lahore Pakistan and Silymarin of same purity from Abbot Laboratories, Karachi, Pakistan, while TQ was purchased from Sigma-Aldrich (USA). All the chemicals and reagents used in this study are of analytical grade.

Preparation of Experimental Animals and Grouping: Fourty (40) Male adult albino rabbits of same breed and of 4 months of age was purchased from the animal house of University of Veterinary and Animal Sciences, Lahore, Lahore. They were further kept in animal house of Johar Institute of Professional Studies, Lahore for acclimatization before the start of experimental procedure. They were kept under the standard control temperature (25±3) and humidity. They were kept under natural light and dark cycle. All rabbits were fed on standard diet and water *ad libitum***.** Rabbits were randomly divided in five groups, each group contains 8 rabbits **(Table 1)**.

Table 1: Grouping of Rabbit Models

Group 1: Normal Control	**Routine diet along with water ad-libitum for 0-28 days.**
Group 2: Treated with hepatotoxic drugs	**Routine diet + Isoniazid (50mg/kg body weight) + Rifampicin (250mg/kg body weight) P.O. for 0-28 days**

	as hepatotoxic drugs.
Group 3: Treated with hepatotoxic + standard hepatoprotective drug.	**Routine diet + Isoniazid (50mg/kg body weight) + Rifampicin (250 mg/kg body weight) + Silymarin (100mg/kg body weight) P.O. for 0-28 days.**
Group 4: Treated with hepatotoxic drugs + Thymoquinone (TQ)	**Routine diet + Isoniazid (50mg/kg body weight) + Rifampicin (250 mg/kg body weight) + Thymoquinone (20mg/kg body weight) P.O. for 0-28 days.**
Group 5: Treated with hepatotoxic drugs + Thymoquinone (TQ)	**Routine diet + Isoniazid (50mg/kg body weight) + Rifampicin (250 mg/kg body weight) + Thymoquinone (40mg/kg body weight) P.O. for 0-28 days.**

Blood sampling: Blood from all the rabbits was collected in heparinized tubes (for hematological analysis) and in simple glass tubes (for the collection of serum). 5 blood samples were drawn from each rabbit on day 0, 7, 14, 21, 28. All rabbits were sacrificed 24hours after the last treatment using vapours of light ether and their liver was collected and preserved in 10 % formalin for further histopathological analysis. Euthanasia was done according to the American Veterinarian Medical Association (AVMA) Guidelines for Euthanasia (2013 Edition **[19]**. Blood collected for serum extraction after half an hour put into the centrifuge machine for centrifugation at 2000-3000rpm for 2-3 minutes. After 2-3 minutes. Serum was collected in labeled eppendorf tubes by using micropipette and stored at -80^{0}C for further use.

Biochemical Analysis: Liver function was assessed by calculating serum AST (aspartate aminotransferase) and ALT (alanine aminotransferase) by chemistry analyzer using commercially (randox) available biochemical kits.

Histopathological analysis:

Previously preserved liver in 10% formalin was cut in to small pieces of approximately 5 mm in dimensions using surgical blade. Pieces were washed for 10 to 12 hours with distilled water. They were washed further by dipping in 70% of diluted ethanol for the time period of 10 hours. After that these pieces were again washed with 85% and 95% of alcoholic dilution for 4-6 hours respectively. Then these pieces were dipped in to absolute alcohol solution I for at least 2 hour and the same procedure was repeated through dipping this solution again in absolute alcohol solution II. After that, the xylene and alcohol solution was used for dipping these liver pieces for at least 2 hours. Then these pieces were dipped in Xylene I solution and Xylene II solution. In each solution these pieces were dipped for at least 30 minutes. After that these were dipped in paraffin I, paraffin II and paraffin III and in each solution the pieces were dipped for 2 to 4 hours. Melted wax was used to mold the liver pieces in wax.The molded cool wax was cut in to uniform size slices, each of 5 micron

meter diameter through an advance cutting machine Micron HM 325 and these fine slices were fixes on the glass slides. For staining the slides the *Haemotoxylin & Eosin* stain (H & E stain) were used. These stained slides were finally examined under microscope. Olympus PM – 10ADS automatic light microscope (Olympus optical Co., Tokyo, Japan) with a 20X and 40X objective lenses equipped with calibrated ocular micrometer.

Statistical analysis: The data was expressed as Mean ± SD. Statistical analysis was performed using Graph-pad Prism software. One-way *ANOVA* applied followed by post hoc Tukey test and P value equal or less than 0.005 was considered significant.

RESULTS AND DISCUSSION

The purpose of this study is the use of natural and safe medication to increase the therapeutic effects of the antituberculosis drugs and reducing their harmful adverse effects. Thymoquinone is administered in the test groups along with the isoniazid and rifampicin to evaluate if co-administration of thymoquinone, which is found safer and beneficial for the patient health in terms of hepatotoxicity. Medical research concluded that anti-oxidant properties of Thymoquinone are responsible for the hepatoprotective as well as anti-inflammatory effects observed in the groups treated with Thymoquinone **[20]**. Thymoquinone and Hydro-thymoquinone both have antibacterial properties and can be used in combination with other antibiotics **[21]**. Thymoquinone has antibacterial property as well as it is hepatoprotective. In tuberculosis therapy isoniazid and rifampicin are used for more than 6 months until the patient is cured so our purpose is to study the hepatoprotective effects of Thymoquinone if used in combination with Isoniazid and Rifampicin. The results show that increased dose of thymoquinone results in decreasing the value of ALT and AST towards the normal range. The group of rabbits that are treated with isoniazid and Rifampicin show increased value of ALT and AST, this means that the combination of Thymoquinone with Isoniazid and Rifampicin is proved to be very effective in normalizing the values of liver function test. Histopathological view of hepatocytes of the rabbits that are treated with isoniazid and rifampicin shows extreme hepatocytes degeneration and the Hepatocytes of group 4 and group 5 rabbits shows normal hepatocytes. We suggest that thymoquinone is more beneficial to be used in combination with Isoniazid and Rifampicin as it minimize the risk of hepatotoxicity.

Table-2: Serum levels of ALT (U/L) in all the groups from day 0 to 28th days in all the groups (n=8)

Day	Groups				
	Control	INH+RIF Treated	INH+RIF+ SILYMARIN	INH+RIF+ TQ (20 mg/kg)	INH+RIF+TQ (40 mg/kg)
0	18.13	16.75	17.50	17.75	18
7	17.63	60.00	18.13	20.50	19.75
14	19.38	64.00	17.63	18.63	16.88
21	15.38	70.33	15.57	16.13	15.75
28	15.00	76.50	20.63	15.38	15.03
Mean	17.1	57.52###	17.89***	17.678***	17.08***
SD	1.865	23.64	1.814	1.8194	1.6725

Represents P < 0.001 as compared with control group while *** represents P < 0.001 as compared with INH+RIF treated group

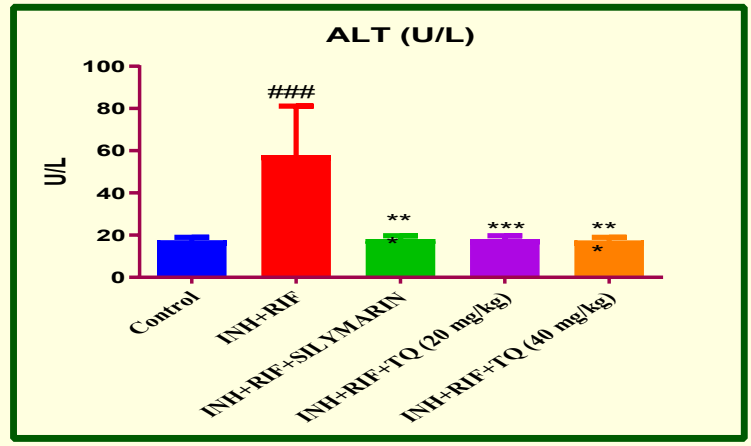


Figure 1: mean values of ALT and SD in different groups

Table 3: Serum AST level (U/L) in all the groups from day 0 to 28th days in all the groups (n=8)

Days	Groups				
	Control	INH+RIF Treated	INH+RIF +SILYMARIN	INH+RIF+TQ (20 mg/kg)	INH+RIF+TQ (40 mg/kg)
0	47.63	49.75	48.57	47.63	48.38
7	55.13	69.67	70.00	55.63	49
14	51.63	72.67	59.00	50.13	50.25
21	56.00	84.00	56.13	59.25	52.50
28	40.13	89.17	48.00	41.63	51.75
Mean	50.1	73.05#	56.34	50.854*	50.376*
SD	6.48	15.28	8.0454	6.153	1.567

#Represents P < 0.05 as compared with control group while * represents P < 0.05 as compared with INH+RIF treated group

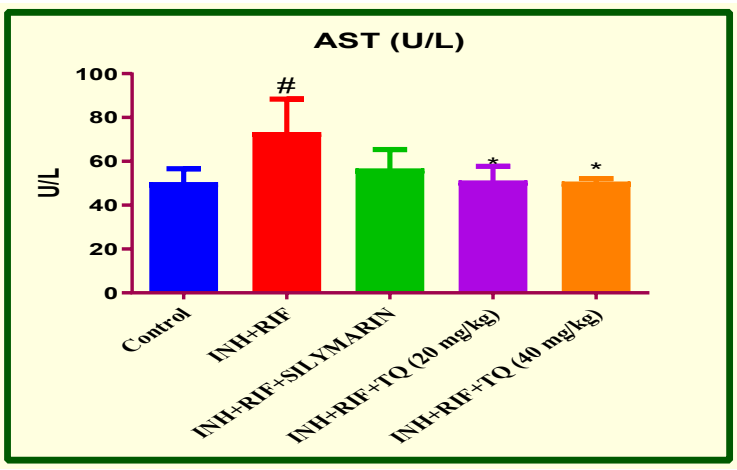
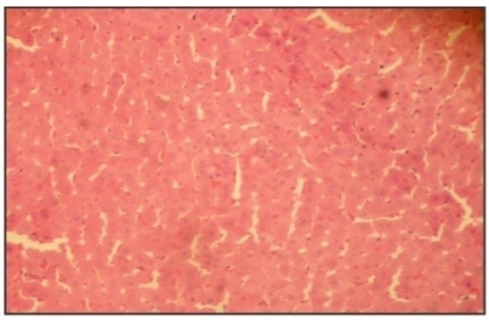
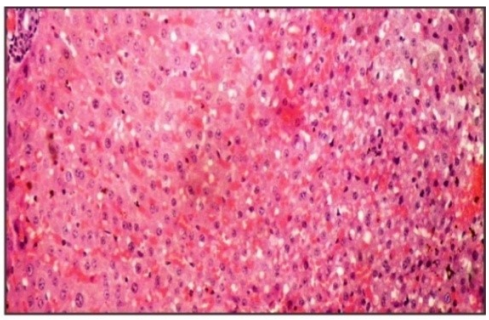


Figure 3: Mean values of AST and SD in different groups

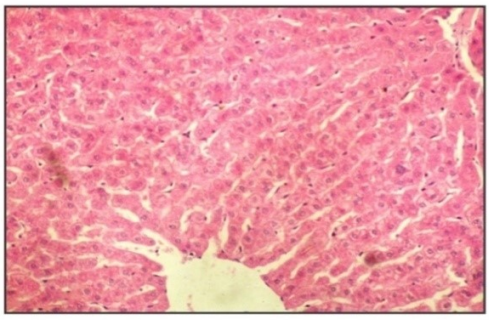
Histopathological Studies of Hepatic tissues



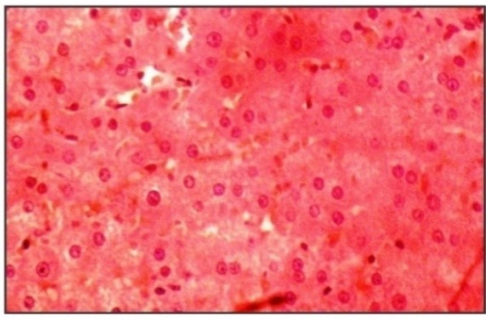
Slide of Hepatocytes obtained from rabbit of control group (H &E, x20)



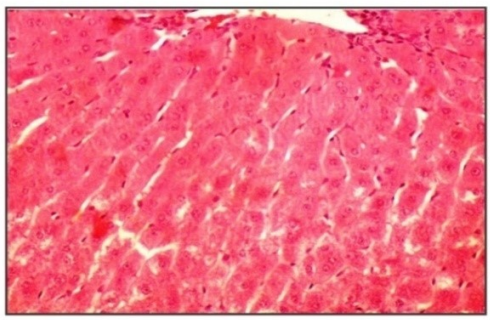
Slide of Hepatocytes from INH+RIF Treated Group (H & E, x 20)



Slide of Hepatocytes from silymarin Treated Group (H & E, x 20)



Slide of Hepatocytes from TQ (20 mg/kg) Treated Group (H & E, x 20).



Slide of Hepatocytes from TQ (40 mg/kg) Treated Group (H & E, x 40)

Figure 4: Histopathology of hepatic tissues

CONCLUSION

This study was designed to evaluate the hepatoprotective effect of Thymoquinone against isoniazid and rifampicin induced hepatotoxicity. 40 albino rabbits were used which were divided in group of five.

Silymarin used as a standard drug. In group 1 routine diet was given to rabbits, in group 2 isoniazid and rifampicin were administered, and in group 3 isoniazid and rifampicin is administered along with Silymarin. Different doses of Thymoquinone administered in group 4 and group 5 to evaluate which has maximum effect concentration of drug. Thymoquinone found improved to counter the hepatotoxic effect induced by anti tuberculosis drugs.

CONFLICT OF INTREASTS

Authors declare no conflict of interests.

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