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Hepatoprotective activity of hydro-alcoholic extracts of *Hygrophila Auriculata* Heine against Isoniazid and Rifampicin Induced Hepatotoxicity in Rats.

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Abstract- Hepatotoxicity can affect hundreds of millions of people worldwide. It is the common non-neoplastic cause of death among hepatobiliary and digestive disorders. Serious side effects, the cost of the modern medicine and improper channel of treatment and competitive efficacy of natural products made the person through the world to look for classical plant drugs for the treatment of hepatotoxicity. In view of the pharmacological and biological properties and chemical constituents of plants from Acanthaceae species, it was decided to study the plant Hygrophilaauriculata which is widely used in folk medicine. In the present study, an attempt is made to find out the extent of hepatoprotective activity in different parts of plants being used. As a first step hepatoprotective activity of Hydro alcoholic extract of Hygrophila auriculata Heine tried at different dose at 100mg/kg and 200mg/kg dose of Hydro alcoholic extract of Hygrophilaauriculata, orally for 28 days to treat induced hepatotoxicity in animal who received 100mg/kg INH &100 mg/kg RIF.in sterile water orally for 28 days. Hepatoprotective property evaluated on different clinical parameters like Liver Enzymes (AST , ALT , ALP), Total Proteins, Total Bilirubin, In this study the result reveals that, the statistically significant difference in biochemical parameters, in toxic control group (Group II), Indicate that hepatic damage has been induced by INH+RIF. Following treatment with Silymarin (100mg/Kg), Low dose (100mg/Kg) & high dose (200mg/Kg) of Hydroalcoholic extract of Hygrophilaauriculata, all the liver marker enzymes such as AST, ALT, ALP, GGT & TB were reduced and total protein restored to normal value.

Key words- Hepatotoxicity, Hepatoprotetive, INH, RIF, AST , ALT , ALP, GGT and TB etc.

Introduction-

Hepatotoxicity[8]

Hepatotoxicity (from hepatic toxicity) is chemical-driven damage. Chemicals that cause liver damage are called hepatotixins. It is possible side effect of certain medications, but can also be caused by chemicals used in laboratories and industry, and natural chemicals, like microcystins.

Types

Hepatotoxicity can be considered to occur in two forms, symptomatic or idiosyncratic.

Drugs or toxins that have a symptomatic hepatotoxicity are those that have predictable

dose-response curve (higher concentrations cause more liver damage) and well characterised mechanisms of toxicity.

In contrast, idiosyncratic hepatotoxins are agents that cause liver damage in only a small fraction of the population that is exposed to the agent, does not have clear dose response or temporal relationship, and most often do not have predictive models.

Hepatic metabolism

Many common drugs are metabolised by the liver in significant amounts. This, together with its role as first filter of blood loaded with substance absorbed from the gut, makes hepatotoxicity one of the main concerns. All lead optimisation cascades must deal in some way with the issue of hepatic toxicity. Enterohepatic circulation is an especially thorny issue in drug discovery.

Drug induced liver injury

Drugs are an important cause of liver injury. More than 900 drugs, toxins, and herbs have been reported to cause liver injury, and drug account for 20-40% of all instances of fulminant hepatic failure. Approximately 75% of the idiosyncratic drug reactions result in liver transplantation or death. Drug-induced hepatic injury is the most common reason cited for withdrawal of an approved drug. The manifestation of drug-induced hepatotoxicity adr highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure.

Acetaminophen and hepatotoxicity

One of the main cause of drug induced hepatotoxicity in western countries is acetaminophen (paracetamol, also known by the brand name Tylenol®) poisoning, which is a symptomatic hepatotoxin. Hepatic damage can sometimes be detected at advanced stages by the typical yellow skin (jaundice) that arises from defective bilirubin liver metabolism. For earlier stages, there are a number of convenient liver function tests. Acetylcysteine can limit the severity of the liver damage by capturing the toxic acetaminophen metabolite.

Risk factor for drug-induced liver injury

A number of risk factors predispose an individual to heapatic drug injury such as pre-existing liver damage, ageing, female sex and genetic inability to perform particular biotransformation. Emergence of such liver complications caused due to hepatotoxic agents like Alcohols, CCL_4, Bacterial toxins etc.

Pathophysiology and mechanisms of drug-induced liver injury

The following are some of the mechanisms that have been described:
-Disruption of the hepatocyte.
-Ddisruption of the transport proteins. -Clytolytic T –cell activation
-Apoptosis of hepatocytes
-Mitochondrial disruption
-Bile duct injury
-Drug toxicity mechanisms
-Intrinsic or predictable drug reactions
-Idiosyncratic drug reactions
-Hypersensitivity
-Metabolic-idiosyncratic

Anti-tubercular drugs and liver toxicity

In India, pulmonary tuberculosis is one of the major cause for adult deaths.[10] INH and RIF, the first line drug used for tuberculosis chemotherapy, are associated with hepatotoxicity.[11]The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8%-30%) compared to that in western countries (2%-3%) with a similar dose schedule.[12]

INH acetylated metabolites that are thought to be hepatotoxins.[25]Oxidative stresses as one of

the mechanism for INH+RIF-induced hepatic injury.[27]

Majority of normally formed free radicals are removed by the action of reduced glutathione on reduction results in the initiation of lipid peroxidation (LPO) resulting in tissue injury.[28]Liver injury has been observed when glutathione stores are markedly depleted.[29]

Herbal formulation for Hepatoprotective effect.

Around 1250 plants are currently used in various Ayurvedicpreparation.

Materials and Methods

Chemicals

For this study, a R-CINEX Capsule containing RIF 450 mg and INH 300 mg, manufactured by Lupin Pharmaceuticals Ltd. was used to induce hepatotoxicity in rats at a dose of 100 mg/kg daily for 28 days.[65] The capsules were dissolved in distilled water and given orally by feeding needle.

Extraction of Hygrophila auriculata (Schumach.) Heine:

The whole plant of hygrophilaauriculata was dried in the shade. Then the shaded dried plants were powdered to get coarse powder. And about 500 gms of the dried coarse powder of hygrophila auriculata were soaked in the extractor and macerated for 30 hrs with petroleum ether, followed by Chloroform. Then it is extracted with Hydro alcohol (30:70) by continuous hot percolation technique using Soxhlet apparatus for 72 hrs. Crude extract were distilled under vacuum condition. After concentration, the ethanol extract gives brownish residue which weighs about 7.2 gms. This extract was used for evaluation of hepatoprotective activity.

Animals

Male wistar albino rats (150-200 gms) were produced from animal experimental laboratory, and used throughout the study. They were housed in micronylon boxes in a control environment (temp 25± 2^0c) and 12 hrs dark/light cycle with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice , the rats were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygiene environment in our animal house.

Induction of Hepatotoxic in Animal Model

a) Hepatotoxic induced by ethanol
b) Hepatotoxic induced by paracetamol
c) Hepatotoxic induced by CCl_4
d) Hepatotoxic induced by INH+RIF

a) Hepatotoxic induced by Ethanol

The basic mechanism in the induction of Hepatotoxic by ethanol is that ethanol is principally metabolized to acetaldehyde in the liver and Seldom in other tissue by alcohol dehydrogenase as well as CAT (Catalase). Acetaldehyde is further oxidized into acetate by acetaldehyde dehydrogenase oxidase, leading to the generation of ROS/free radical. Ethanol is also oxidized by a microsomal ethanol oxidizing system ($CYP2E_1$ mainly) to acetaldehyde and 1-hydroxyethyl radical especially following chronic ethanol consumption by which $CYP2E_1$ is induced. Excessive alcohol intake results in disequilibrium in iron homeostasis & iron overload which further enhance oxidative stress by catalyzing the formation of more noxious hydroxyl free radical. Hence induction of $CYP2E_1$ and iron overload by ethanol are critical path way by which ethanol generates a state of oxidative stress in hepatocytes.[60]

b) Hepatotoxic induced by Paracetamol

The mechanism by which over dosage with paracetamol leads to hepatocellular injury & death

involves its conversion to the toxic NAPQI (N-acetyl–para benzoquinone imines) metabolite. The glucoronidesulfa conjugation pathways become saturated and increasing amounts undergo CYP-mediated N-hydroxylation to form NAPQI. This is eliminated rapidly by conjugation with GSH & then further metabolized to a mercapturic acid and excreted into urine. In the setting of paracetamol overdose, hepatocellular levels of GSH become depleted. The highly reactive NAPQI metabolite binds covalently to cell macromolecules leading to dysfunction of enzymatic system and structural & metabolic disarray further more depletion of intracellular GSH renders the hypotocytes highly susceptible to oxidative stress and apoptosis.[61]

c) Hepatotoxic induced by CCl_4

CCl_4 induces liver damage by producing free radical intermediates.[62] CCl_4 is converted to tri chloromethyl radical (Ccl_3) by the P-450 system. Which in turn is converted to peroxy radical ($Ccl_3 o_2$), which causes the damage.[63]

d) Hepatotoxic induced by INH+RIF

During metabolism of INH, Hydrazine can be produced by both directly (from INH) and indirectly (from acetyl hydrazine). The direct pathway involves hydrolysis of the amide bond of INH to produce isonicotinic acid and hydrazine. The indirect pathway involves acetylation of INH to acetyl-INH by N-acetyl transferase, hydrolysis of acetyl INH to isonicotinic acid and acetyl hydrazine, and hydrolysis/deacetylation to hydrazine. Hydrazine is a known hepatotoxins, positive correlation between plasma hydrazine levels and severity of INH-induced hepatocellular damage in rabbits.[64]

RIF is reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell Lipid Peroxidation, and cytochrome P-450 was thought to be involved in the synergistic effect of RIF on INH.

Experimental Model

For the study of hepatoprotective activity, on experimental model is selected in such a way that it would satisfy the following condition;

- The animal should develop liver toxicity rapidly and reproducibly.
- Pathological changes in the site of induction should result from liver damage.
- The symptoms should be ameliorated or prevented by a drug treatment effective in human beings.
- The drug tested should be administered orally.
- Drug dosage should approximate the optimum therapeutic range for human, scaled the test animal weight.

Treatment Protocol

The animal were groups into five groups of six animals each , Maintained on standard diet and water *ad libitum*

Group 1: Served as Normal Control received 10ml/kg Normal Saline for 28 Days , orally

Group 2: Served as toxic control , received 100mg/kg INH &100 mg/kg RIF in sterile water orally for 28 days.[63]

Group 3: Served as the Positive Control received 100mg/kg of Silymarin orally for 28 days.[64]

Group 4: Served as Treatment Control received 100mg/kg of Hydro alcoholic extract of Hygrophilaauriculata, orally for 28 days.[65]

Group 5: Served as Treatment Control received 200mg/kg of Hydro alcoholic extract of Hygrophilaauriculata, orally for 28 days.

Evaluation of clinical parameters

1) Liver Enzymes (AST,ALT, ALP)

Determination of certain Serum enzymes is considered useful in various types of liver injury, wheather hepatocellular or Cholestatic, as well as in quantifying liver damage.

Serum aspartate transaminase (AST) is a mitochondrial enzyme released from heart, liver, skeletal muscle and Kidney and Serum transaminase (ALT) is a cytosolic enzyme primarily present in the liver.

Serum levels of AST and ALT are increased on damage to the tissue producing them. Thus serum estimation of ALT which is fairly specific for liver tissue is of greater value in liver cell injury, whereas AST level may rise in acute necrosis or ischaemia of other organs such as the myocardium, besides liver injury. Alcoholic liver disease is associated with mild to moderate elevation of transaminase.[66]

2) Total Proteins

Liver cells Synthesize albumin, fibrinogen, Prothrombin, hepatoglobin, transeferin, alpha fetoproteins and acute phase reactant proteins. The blood level of these plasma proteins are decreased in extensive liver damage. Hypoalbuminaemia may occur in liver disease having significant destruction of hepatocytes. Hyperglobulinaemia may be present in chronic inflammatory disorders in chronic hepatitis.[67]

3) Total Bilirubin

Bile is produced by the liver, stored in the gall bladder and secreted via biliary ducts into the duodenum. Bile consists of biliary phospholipids and primary and secondary bile acids. To understand the mechanisms underlying biliary pathology, it is important to understand bilirubin metabolism. In brief, jaundice will develop if bilirubin is excessively produced, or there is impaired hepatic uptake and conjugation of bilirubin or it is insufficiently excreted in duodenum.[68]

Statistical analysis

All results were expressed as Mean ± SEM (n=6). The data were analysed by one –way ANOVA; statistically significant effects were compaired using Newman Keul'sMultiple range test. Statistical significant was determined at $p < 0.01$.

Results

Administration of INH & RIF combination only, showed a significant derangement of Liver function as assessed by change in serum enzymes such as AST, ALT, ALP, GGTP, TP & TB, as well as liver histopathology table no 2

Effect of body weight of animal

INH & RIF treated group showed a significant decrease in body weight compared to normal control group (Group I). Similarly, rats treated with high dose of Hydroalcoholic extract of Hygrophilaauriculata (Group V) showed a significant reduction in body weight when compare to Control group (Table no-2). However in groups (III & IV) treated animals no significant change in body weight.

Assessment of Serum Marker Enzymes

Hepatic damage induced by INH & RIF causes significant rise in Liver marker enzymes AST, ALT, ALP, GGT & TB and Significant decrease in liver marker enzyme such as Total protein in Group II treated animals. (329.58 ± 3.46 , 150.13 ± 3.12 , 230.81 ± 2.85 , 130.45 ± 2.41 , 1.71 ± 0.18 & 4.14 ± 0.29) respectively, where as in treated groups (Group IV & V) there was significant decrease in AST, ALT, ALP (256.65 ± 2.53 , 117.88 ± 3.85 , 192.6 ± 1.48) , (235.66 ± 2.10 ,103.38 ± 1.98 , 171.75 ± 1.89) respectively , also significant decrease in GGT & Total Bilirubin (75.34 ± 2.47 , 0.94 ± 0.044) , (Group IV) (59.10 ± 1.60 , 0.73 ± 0.032) (Group IV) respectively. In both groups there was significant

increase in the level of Total protein (5.91 ± 0.13 & 5.8 ± 0.29) respectively as compare to the toxic control (Group II).

In Group-III (positive control) also have significant decrease in AST , ALT , ALP (263.88 ± 4.16 , 99.41 ± 2.67 , 158.45 ± 2.35), accompanied by significant increase in level of total protein (6.6 ± 0.25) and also significant decrease in GGT & TB (54.4 ± 1.95 & 0.68 ± 0.045) as compare to the toxic control (Group II)

Histopathology

Normal liver Morphology was observed in control animals with evidence of Portal inflammation, ballooning degeneration or fatty change and necrosis. However, histopathological changes were observed in all other treatment groups.

Portal inflammation

In (Group II) INH & RIF treated animals showed presence of portal inflammation. However, groups treated with Silymarin (Group III) both doses of Hydroalcoholic extract of Hygrophilaauriculata showed a significant reduction in inflammation compare to INH & RIF treated groups (Group II).

Ballooning degeneration

Moderate to severe ballooning degeneration was observed in all the rats in INH & RIF treated animals (Group II). In other treated groups, all rats showed ballooning degeneration, but with severity. However, reduction in severity compare to INH & RIF groups was statistically significant inSilymarin treated groups (Group III) & both doses of H.A. treated groups.

Fatty change All the treated groups showed moderate to severe fatty changes except for high dose of H.A. (200mg/Kg) treated groups in which none of rats showed fatty changes and this difference was highly significant ($P < 0.001$) compared withINH& RIF treated animals (Group II).

Necrosis

Moderate to severe necrosis was observed in 4 out of 6 rats in INH & RIF treated animals in (Group II). However, necrosis was significantly absent in all the rats treated with Silymarin and both doses of H.A. as compared with INH & RIF treated animals in (Group II).

Effect of Hydoro alcoholic extract of Hygrophilaauriculata on biochemical Parameters of INH-RIF intoxicated rats.

Table No.1

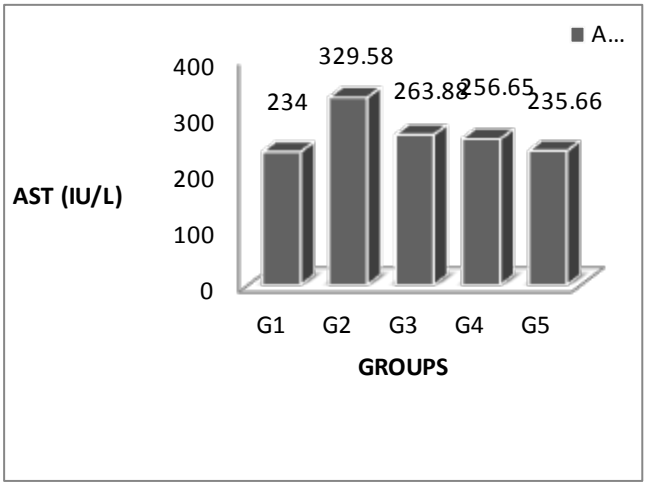
Groups	AST IU/L	ALT IU/L	ALP IU/L	GGTP IU/L	TP gm/dL	TB mg/dL
Group I (G1)	234.0 ±3.09	77.35 ±2.26	134.25 ±1.86	34.18 ±1.76	6.90 ±0.30	0.39 ±0.055
Group II (G2)	329.58 *a ±3.46	150.13 *a ±3.12	230.81 *a ± 2.85	130.45 *a ± 2.91	4.14 *a ± 0.29	1.71 *a ±0.18
Group III (G3)	263.88 *b ±4.16	99.41 *b ±2.67	158.45 *b ± 2.35	54.4 *b ±1.95	6.6 *b ±0.25	0.68 *b ± 0.045
Group IV (G4)	256.65 *b ±2.53	117.88 *b ±3.85	192.6 *b ±1.48	75.34 *b ±2.47	5.91 *b ±0.13	0.94 *b ±0.044
Group V (G5)	235.66 *b ±2.10	103.38 *b ±1.98	171.75 *b ±1.89	59.10 *b ±1.60	5.8 *b ± 0.29	0.73 *b ± 0.032

G1- Normal Control; **G2**- Toxic Control; **G3**- Standard control (Silymarin); **G4**- Treatment group 100mg/kg; **G5**- Treatment group 200mg/kg.

- Values are expressed as Mean ± SEM.
- Values were find out by using ONE WAY ANOVA followed by Newman Keul's multiple range test.
- *a values were significantly different from normal control (G 1) at (P<0.01)
- *b values were significantly different from toxic control (G 2) at (P<0.01)

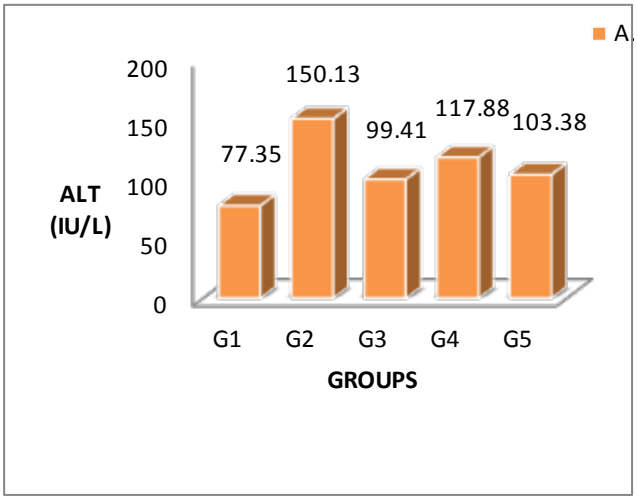
Serum levels of AST Values of means of 6 rats in each group.

Graph No.1

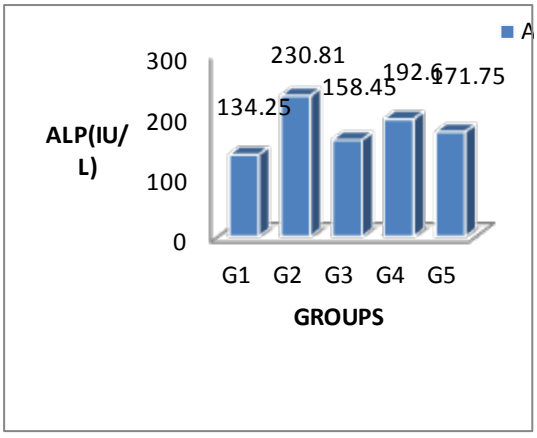


Serum levels of ALT Values of means of 6 rats in each group.

Graph No.2

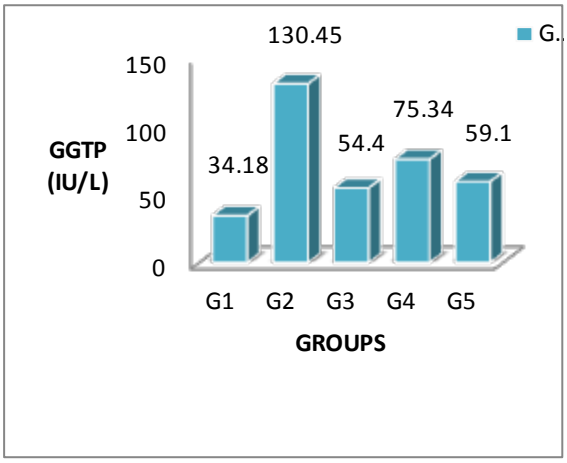


Serum levels of ALP Values of means of 6 rats in each group. Graph No.3



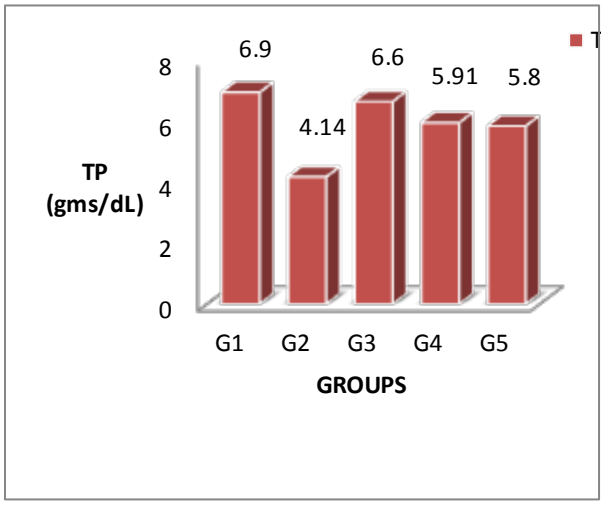
Serum levels of GGTP Values of means of 6 rats in each group.

Graph No.4



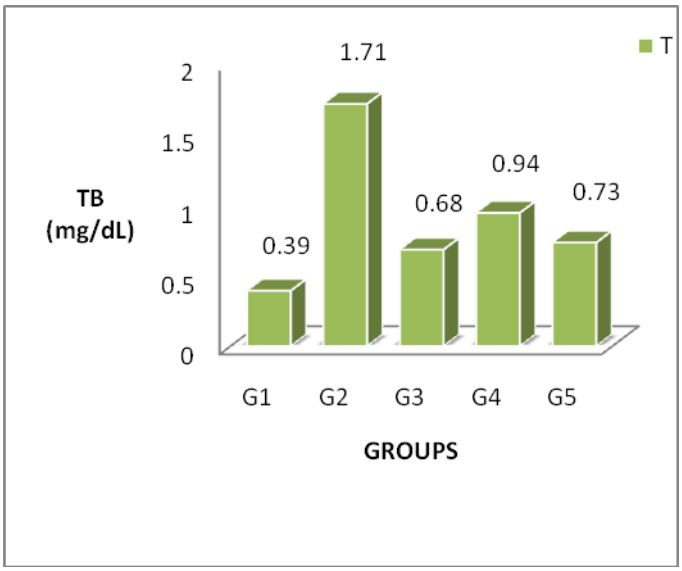
Serum levels of TP Values of means of 6 rats in each group.

Graph No.5



Serum levels of TB Values of means of 6 rats in each group.

Graph No.6



Discussion

Selection of an animal model is important, if the results of the experiment are to be extrapolated to a human population. Looking at the comparative biology of test species for acetylation,deacetylation, debrisoquine oxidation , hepatic epoxide hydrase& β-glycuronidase activators, it is evident that the rats mimics human biology more closely than any other species.[69,70] In the present study, a hepatotoxic model of rat was successfully produced by the drug 100 mg/Kg INH &100 mg/Kg RIF (B.W) orally for 28 days. This dose is somewhat higher than used by other workers, and much higher than used by other workers, and much higher than comparable human dose. But , this is justified by taking into account the fact that rat liver is most resistant to injury among rodents and that rodents are fast metabolizers.

During the metabolism of INH, hydrazine is produced directly (from INH) and indirectly (from acetyl hydrazine). From our study , it is evident that hydrazine plays a role in INH induced liver damage in rats, which is consistent with the report by Sarich et al[71], who found the severity of INH- induced hepatocellular damage had a positive Correlation with plasma hydrazine levels in rabbits.

INH is metabolized in the Liver primarily by acetylation and hydrolysis, and it is these acetylated metabolites that are thought to be hepatotoxins.[72] Previous report in rats suggest that the hydrazine metabolite of INH and is subsequent effect on $CYP2E_1$ induction is involved in the development of INH-induced hepatotoxicity[73], and also oxidative stress as one of the mechanism for INH-RIF induced hepatic injury[74].

In this study the result reveals that, the statistically significant difference in biochemical parameters, in toxic control group (Group II), Indicate that hepatic damage has been induced by

INH+RIF. Following treatment with Silymarin (100mg/Kg), Low dose (100mg/Kg) & high dose (200mg/Kg) of Hydroalcoholic extract of Hygrophilaauriculata, all the liver marker enzymes such as AST, ALT, ALP, GGT & TB were reduced and total protein restored to normal value.The estimation of GGTP (Gamma glutamyltranspeptidase) levels is a valuable screening test with high predictive value for liver disease[75]. A number of drugs & chemicals are known to increase GGTP activity by the induction of hepatic microsomal enzymes[76].

In this study the result also reveals that treatment with Silymarin (100mg/Kg), low dose (100mg/Kg) & high dose (200mg/Kg) of Hydroalcoholic extract of H.A. groups significant changes occurred in GGTP levels. This suggests that the extracts possess significant hepatoprotective activity

Histopathological observations of Liver sections from the control group showed normal cellular architecture with distinct hepatic cells, Sinusoidal spaces and a central vein(Fig. No.4). In contrast the INH+RIF groups revealed the most severe damage of any of the groups; the liver sections showed massive fatty changes, necrosis, ballooning degeneration, broad infiltration of lymphocytes, and the loss of cellular boundaries (Fig. No.5 & 5a)

The liver sections of the rats treated with Hydroalcoholic extract of H.A. (Fig. No. 7, 7a, 8, & 8a) showed a more & less normal lobular pattern with a mild degree of fatty change, necrosis, and Lymphocyte infiltration almost comparable to the control (Fig. No. 4) and the Silymarin treated group (Fig. No.6 & 6a).

Conclusion

In the present study, Hydoalcoholic extract of Hygrophilaauriculata possessed strong hepatoprotective activity in rat Model of INH + RIF Induced. The hepatoprotective activity of H.A. may be due to its free-radical scavenging activity, resulting from the presence of some flavonoids and phenolic compounds in the extacts. Additional studies are in progress to better understand the mechanism of action of Hygrophilaauriculata that is responsible for the hepatoprotectve activity.

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