HEPATOPROTECTIVE ACTVITY OF ETHANOLIC AND AQUEOUS EXTRACT OF *TERMINALIA BELERICA* IN RATS

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Summary

Hepatoprotective activity of the ethanolic and aqueous extracts of Terminalia belerica Roxb. of the family Combretaceae was studied in Wistar rats using the ethanol-induced liver hepatotoxicty. Ethanol administration resulted in significant elevation of physical parameters (Viz. rat liver weight and liver volume), biochemical parameters like (Viz. serum aspirate transaminase (AST); alanine transminase (ALT); alkaline phasphatase (ALP); direct bilirubin and total bilirubin levels, while albumin and total protein were found to be decreased compared to normal group). Pretreatment with silymarin, ethanolic extract of Terminalia belerica (ALTB) and aqueous extract of Terminalia belerica (AQTB) significantly prevented the physical and biochemical changes induced by these hepatotoxins. Histopathology of liver confirmed our finding as the treatment with the extracts resulted in minor liver cell damage compared to toxic control group. The hepatoprotective effect offered by ALTB (400 mg/kg, p.o.) was found to be significantly greater than AQTB (400 mg/kg, p.o.) and standard (silymarin 50 mg/kg, p.o.) group.

Keywords: *Terminalia belerica*, Biochemical parameters, Hepatoprotective, Alcoholic Liver Diseases (ALD), Ethanol.

Introduction

The liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction. (Ward and Daly, 1999)

Liver disease has become a global concern worldwide. The principal causative factor is increasing ethanol consumption, infection, malnutrition, anemia and availability of hepatotoxic drugs over the counter. Treatment options for common liver diseases such as cirrhoses, fatty liver and chronic hepatitis are problematic (Petry, 2001). In view of lack of synthetic agents (there is no single effective allopathic medication) for the treatment of hepatic disorder, there is a growing focus to evaluate traditional herbal medicines for hepatoprotective activity (De *et al*., 1993). Therefore; there is a need to develop satisfactory hepatoprotective drugs.

In the case of alcoholic liver disease (ALD), no such comprehensive animal model currently exists (Amin *et al.,* 2003). In the absence of a comprehensive model, ALD studies using animals are designed to answer specific questions about different aspects of the disease, usually addressing only one or two experimental variables at a time while holding others constant. Consequently, it was through interesting to standardized a model using alcohol as hepatotoxic agent and to screen various indigenous drugs for their hepatoprotective action.

Terminalia belerica Roxb. (Combretaceae) commonly known as *Bahera* is one of the ingredients of Ayurvedic purgative medicament "*Triphala"*. Traditionally the fruits of this plant were reported greater therapeutic value in the treatments of liver disorders and indigestion (Nadkarni, 1954). The fruits of *Terminalia belerica* were also reported to have purgative (Chakravarti and Tyal, 1947), cardiac depressant, choleretic effect

(Siddiqui, 1994), and antimicrobial activity (Nandy *et al*., 1997). So the present study was undertaken to evaluate the hepatoprotective activity of fruit of this plant.

Material and Methods

Plant Material

The fruits of *Terminalia belerica* were procured from the Natural Remedies Private Limited, Bangalore, India. The Plant material was authenticated by Dr. H.B. Singh, Head, Raw Material Herbarium & Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. The fruits were shade dried at room temperature for 10 days and powdered with the help of a hand –grinding mill and the powdered was passed through sieve no. 60.

Drugs, Chemicals and Biochemical Kits.

Drug like silymarin (Micro labs, Bangalore) was used. Chemical like anesthetic ether (CDH, Mumbai) was purchased through local dealers. Other chemicals and reagents used for extraction and phytochemical analysis were of LR grade obtained locally. For estimation of biochemical parameter; biochemical kits like AST, ALT, ALP, albumin, total protein, direct bilirubin and total bilirubin were obtained from Span Diagnostics Ltd. Surat, India.

Animals

Wistar albino rats (150-200g) purchased from Bioneeds, Nelamangala, Tumkur were maintained in the animal house of PES College of Pharmacy, Bangalore for experimental purpose. Then all the animals were acclimatized for seven days under standard husbandry conditions, i.e.; room temperature of 25 ± 1^0 C; relative humidity 45-55% and a 12:12h light/ dark cycle. The animals had free access to standard rat pellet (Pranav Agro Industries Ltd, Bangalore,

India), with water supplied *ad libitum* under strict hygienic conditions. The animals were habituated to laboratory conditions for 48 hours prior to the experimental protocol to minimize any nonspecific stress. The Institutional Animal Ethics Committee of P.E.S. Collage of pharmacy, Bangalore, India, approved the experiment protocol and experiments were performed in accordance with the guidelines provided by Committee for the Purpose of Control and Supervision of Experimentals on Animals (CPCEA).

Preparation of Extracts.

The powdered material was successively extracted with petroleum ether and ethanol in soxhlet's apparatus. Aqueous extract was prepared by maceration process with water for 72 hours. The drug was extracted with each solvent till complete extraction is effected. The solvents were removed under reduced temperature from the concentrated extracts. Each extract was concentrated by distilling off the solvent to obtain the crude extractives. The percentage yield of ether extract, ALTB and AQTB were 2.3 %, 28.5 %, 16.3 %, respectally.

Phytochemical Screening

Preliminary phytochemical screening of the extracts was carried out by the standard procedure (Khandelwal, 2000).

Determination of LD_{50}.

The acute oral toxicity (AOT) of ethanolic and aqueous extract of fruits of *Terminalia belerica* were determined by using nulliparous, non pregnant female albino rats (Wistar strains) weighing between 180-220 g those maintained under standard husbandry conditions. The animals were fasted 12 hrs prior to the dosing. Animals were administered (orally) with single dose of extracts dissolved in 2% w/v acacia and observed for its mortality during 48 hours study period (short term) toxicity. Based on short-

term profile of drug, the dose of the next animals was determined as per as OECD guideline 425. All the animals were also observed for long term toxicity (14 Days). The LD_{50} of the test extract was calculated using AOT 425 software provided by Environmental protection agency, USA (OECD 2001).

Hepatoprotective activity.

The ethanolic and aqueous extracts were evaluated for their hepatoprotective activity using ethanol-induced hepatotoxicity.

Female/Male adult albino rats (Wistar strain) weighing between 180-220 g were selected for the experimental study. They were dividing into 7 groups, each group consisting of 6 rats and kept under standard laboratory conditions. They had free access to a commercial pellet diet and water *ad libitum*. The room temperature was maintained 25 ± 1^{o}C.

Treatment protocol

Group 1: **Normal group**: animal of this group receive distilled water (6 ml/kg, p.o.) for 30 days.

Group 2: **Control group**: animal of this group received vehicle (2 % acacia, 1 ml/kg, p.o.) and ethanol (3ml/100g/day p.o.) for 30 days.

Group 3: **Standard group**: animal of this group receive silymarin (50mg/ kg/day p.o.) and ethnol (3ml/100g/day p.o.) for 30 days.

Group 4: **Test group**: animal of this group receive ethanolic extract of *Terminalia belerica* (200mg/kg/day) and ethanol (3ml/100g/day p.o.) for 30 days.

Group 5: **Test group**: animal of this group receive ethanolic extract of *Terminalia belerica* (400mg/kg/day) and ethanol (3ml/100g/day p.o.) for 30 days.

Group 6: **Test group**: animal of this group receive aqueous extract of *Terminalia belerica* (200mg/kg/day) and ethanol (3ml/100g/day p.o.) for 30days.

Group 7: **Test group**: animal of this group receive aqueous extract of *Terminalia belerica* (400mg/kg/day) and ethanol (3ml/100g/day p.o.) for 30 days

Ethanol (28.5%) was given 3ml/100gm/day in two divided doses after one hour of drugs treatment of Group 2, 3, 4, 5, 6, and 7 for 30 days (Gujrati *et al.*, 2007 and Sharma *et al.*, 1997).

The animals were anesthetized using anesthetic ether. Blood sample were collected by retro orbital puncture method and serum was used for estimation of AST, ALT, ALP, albumin, total protein, total and direct bilirubin. Immediately after the collection of blood, the animals were euthanized with an over dose of ether; their livers removed, washed in saline and the wet liver volume were determined. The liver was washed by normal saline, blotted with filter paper and weighed immediately (Mastuda *et al.*, 1991) The livers were preserved in 10% formalin for histopathological studies.

Statistics

The results have been expressed as mean ± standard error of mean (S.E.M). Difference in means were compared using one way analysis of variance (ANOVA) followed by Tukey Kramer's post hoc test. $P<0.05$ were considered statistically significant

Results

Preliminary phytochemical studies of our study revealed that presence of saponins, phytosterols, fixed oils and amino acids in petroleum ether extract; cardiac glycoside, carbohydrates, phytosterols, saponins, phenolics compounds and tannins in ethanolic extract and cardiac glycoside, carbohydrates, phenolics compounds and tannins, proteins and amino acids in aqueous extract.

Different doses of ethanolic and aqueous extracts were screened for their oral toxicity. No mortality was recorded till 5000 mg/kg with ethanolic and aqueous extracts, hence the extracts were found to be safe upto the dose levels of 5000 mg/kg.

Effect of ALTB and AQTB on ethanol-induced hepatotoxicity:

Administration of ethanol has produced a significant increase in wet liver weight and volume. Rats pretreated with silymarin (50 mg/kg, p.o.), ALTB (400 mg/kg, p.o.) and AQTB (400 mg/kg, p.o.) showed significant decrease in liver weight and volume compared to the toxic control group. [Table No. 1 & Fig. No.1]

Ethanol administration resulted in significant elevated biochemical parameters like AST, ALT, ALP, direct bilirubin and total bilirubin levels, while albumin and total protein were found to be decreased compared to normal group. Pretreatment of with silymarin, ALTB and AQTB significantly prevented the biochemical changes induced by ethanol. The hepatoprotective effect offered by ALTB (400 mg/kg, p.o.) was found to be significantly greater than AQTB (400 mg/kg, p.o.) and standard (silymarin 50 mg/kg, p.o.) group. [Table No. 2]

In normal animals, liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleolus and central vein. In ethanol (3 ml/ 100g, p.o. for

30 days) treated (toxic) animals the sections shows sever degree of liver damage, showing congestion, macrovesicular and microvesicular steatotsis. In ALTB (400 mg/kg, p.o.) treated animals, liver section showing mild congestion, mild inflammation. Overall picture resembles normal liver. In AQTB (400 mg/kg, p.o.) treated animals, the liver sections showing moderate congestion and inflammation. In silymarin (50 mg/kg, p.o.) treated animals, the liver sections showing moderate congestion and inflammation in the periportal region. [Fig No. 2]

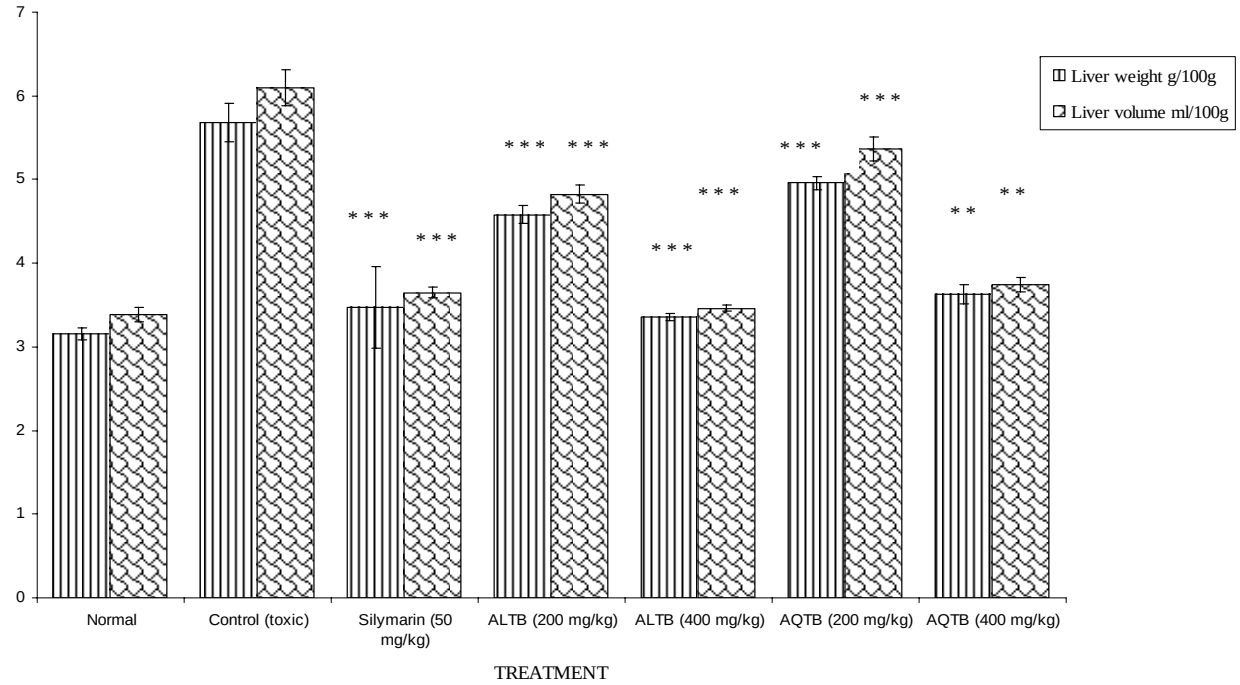
Table: 1. Effect of silymarin, ALTB and AQTB on total liver weight and volume in ethanol-induced liver damage in rats.

SNo.	Treatment	Mean liver weight (g/100g)	Mean liver volume (ml/100g)
1.	Distilled Water (6 ml/kg, p.o.)	3.15 ± 0.07	3.39 ± 0.08
2	2%w/v acacia (1 ml/kg, p.o.) + Ethanol (3ml/100g/bid,p.o.)	5.686 ± 0 .23	6.09 ± 0.21
3	Silymarin (50mg/kg,p.o.) + Ethanol	3.47 ± 0.49***	3.64 ± 0.06***
4	ALTB (200mg/kg, p.o.) + Ethanol	4.57 ± 0.10***	4.8 ± 0.10***
5	ALTB (400mg/kg, p.o.) + Ethanol	3.35 ± 0.04***	3.46 ± 0.04***
6	AQTD (200mg/kg, p.o.) + Ethanol	4.96 ± 0.07**	5.36 ± 0.14**
7	AQTB (400mg/kg, p.o.) + Ethanol	3.62 ± 0.11***	3.74 ± 0.08***
One way- ANOVA	F	67.38	82.91
	df	41	41
	P	0.0001	0.0001

Values are expressed as mean ± S.E.M. n = 6.
** *P* <0.01, *** *P* <0.001 vs. control (Toxic), using one-way ANOVA followed by Tukey Kramer's post hoc test.

ALTB (ethanolic extract of *Terminalia belerica*), AQTB (aqueous extract of *Terminalia belerica*).

Fig: 1. Effect of silymarin, ALTB and AQTB on liver weight and wet liver volume in ethanol-induced liver damage in rats.



** $P < 0.01$, *** $P < 0.001$

Table: 2. Effect of ALTB and AQTB on different biochemical parameters in ethanol-induced hepatotoxicity in rats.

SNo.	Treatment	Serum biochemical parameters						
		AST IU/L	ALT IU/L	ALP IU/L	ALB g/dL	TLP g/dL	BILT mg/dL	BILD mg/dL
1.	Distilled Water	89.33 ± 2.00	38.32 ± 1.04	102.73 ± 4.61	4.83 ± 0.07	14.13 ± 0.28	0.16 ± 0.01	0.32 ± 0.01
2.	2%w/v acacia + Ethanol (3ml/100g/bid, p.o.)	373.14 ± 15.47	208.83 ± 4.50	329.14 ± 7.83	2.09 ± 0.07	7.55 ± 0.23	1.15 ± 0.03	1.91 ± 0.05
3.	Silymarin (50mg/kg, p.o.) + Ethanol (3 ml/100g/bid, p.o.)	136.94 ± 4.40***	61.88 ± 3.25***	148.18 ± 4.16***	4.19 ± 0.12***	12.12 ± 0.16***	0.35 ± 0.02***	0.60 ± 0.01***
4.	ALTB (200mg/kg, p.o.) + Ethanol (3 ml/100g/bid, p.o.)	196.38 ± 6.62***	112.62 ± 4.01***	191.6 ± 5.19***	3.2 ± 0.13***	9.15 ± 0.23**	0.79 ± 0.04***	1.15 ± 0.07***
5.	ALTB (400mg/kg, p.o.) + Ethanol (3 ml/100g/bid, p.o.)	131.95 ± 2.75***	52.46 ± 2.25***	143.76 ± 2.98***	4.258 ± 0.11***	12.42 ± 0.16***	0.36 ± 0.019***	0.59 ± 0.02***
6.	AQTD (200mg/kg,p.o.) + Ethanol (3 ml/100g/bid, p.o.)	227.69 ± 3.49***	146.74 ± 4.13***	300.36 ± 6.28**	2.4483 ± 0.099	8.28 ± 0.32	0.97 ± 0.03***	1.51 ± 0.06***
7.	AQTB (400mg/kg,p.o.) + Ethanol (3 ml/100g/bid, p.o.)	161.42 ± 5.47***	85.11 ± 5.02***	181.47 ± 3.66***	3.4 ± 0.16***	10.27 ± 0.33***	0.73 ± 0.43***	0.97 ± 0.02***
One way- ANOVA	F	255.85	203.39	262.63	79.80	87.93	116.69	108.96
	df	41	41	41	41	41	41	41
	P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are expressed as mean ± S.E. M. n = 6.
*** *P* <0.001 vs. control (Toxic), using one-way ANOVA followed by Tukey Kramer's post hoc test.

ALTB (Ethanolic extract of *Terminalia belerica*); AQTB (Aqueous extract of *Terminalia belerica).* AST (aspirate aminotransferase), ALT (alanine aminotransferase), ALP (alkaline phosphate), ALB (albumin), TLP (total protein), DBIL (direct bilirubin), TBIL (total bilirubin.

Fig: 2. Sections stained with hematoxylin and eosin (H&E 400 X) displaying the liver sections of rats treated with normal, control (toxic), ALTB, AQTB and silymarin in ethanol induced liver damage in rats.

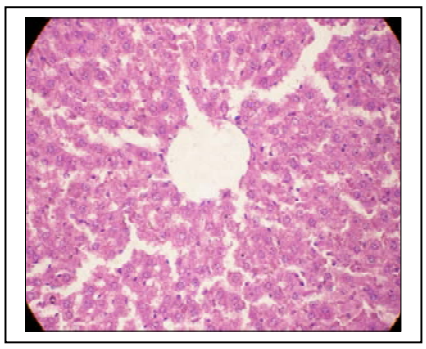


Fig:2 [A] Normal histology of liver

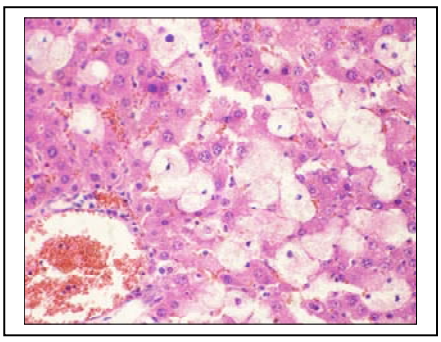


Fig:2 [B] Control (toxic) group

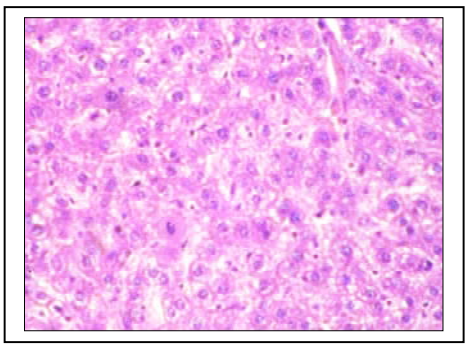


Fig: 2 [C] ALTB (400 mg/ kg,p.o.)

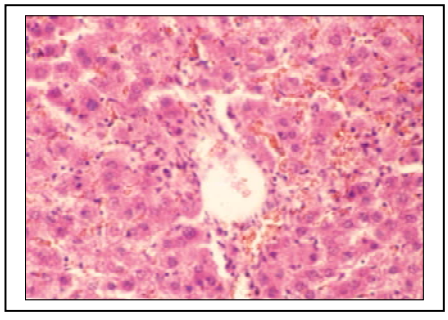


Fig: 2 [D] ALTB (400mg/kg,p.o.)

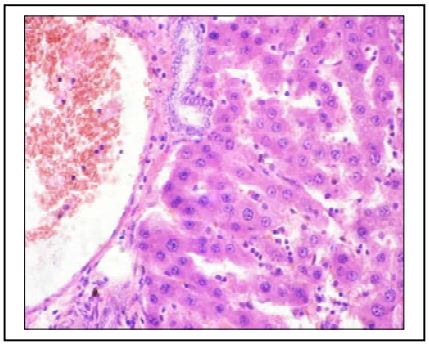


Fig: 2 [E] Silymarin (50mg/kg,p.o.)

Discussion

The liver can be injured by many chemicals and drugs. In the present study ethanol was selected as a hepatotoxicant to induced liver damage, science it is clinically relevant. Ethanol produces a constellation of dose-related deleterious effects in the liver (Leo and Arai, 1982).

Ethanol, even after short-term consumption, induced CYP2E1 enzyme activity in doses that do not causes fatty changes. This enzyme accelerates alcohol metabolism with a resultant increase in acetaldehyde productions (Lieder and DeCarli, 1970). Acetaldehyde is thought to have number of adverse effects like decreased transport and secretion of proteins owing to tubulin polymerization, enhanced vitamin metabolisms and traces metals, which lead to fatal liver disorder (Fromenty B, 1997 and McClain, 1980)

In chronic alcoholics, hepatomegaly occur due to accumulations of lipid and proteins in hepatocytes (Ashakumary and Vijayammal, 1939), with an impaired protein secretion by hepatocytes (Vilstrup and Yygstrup, 1995). Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume (Balncho and Gentil, 1990) observed in the present study. Ethanol- induced increased in total liver weight and volume was prevented by pretreatment with *Terminalia belerica* fruit extracts, thus indicating a hepatoprotective effect.

During hepatic damage, cellular enzymes like AST, ALT and ALP present in the liver cells leak into the serum, resulting in increased concentrations (Deb, 1980). The hepatotoxic dose (3 ml/100g/b.i.d. for 30 days) of alcohol produced an elevation in the liver weight & volume and serum AST, ALT, ALP, direct and total bilirubin, while albumin and total protein were found to be decreased compared to normal group. In experimental animals

pretreated with *Terminalia belerica* fruit extracts (ALTB & AQTB) and silymarin, the total liver weight & volumes, AST, ALT, ALP, direct and total bilirubin levels were significantly lowered, while albumin and total protein significantly increased.

Histopathological changes such as sever congestion, macro vesicular and microvesicular steatosis (fatty changes in hepatocytes) were observed in alcohol treated (toxic) groups. Both extracts prevented these histopathological changes, further indicating their hepatoprotective activity. All the histological changes observed were in correlation with the physical and biochemical parameters.

Thus result of present study clearly demonstrate that the various biochemical (serum AST, ALT, ALP, albumin, total protein, direct bilirubin and total protein levels), physical (liver weight & volume) and histopathological alterations produced by alcohol in the serum and tissue were reserved significantly by the pretreatment of extracts of *Terminalia belerica*.

From the results, the hepatoprotective activity of the extracts were in the ordered of ALTB (400 mg/kg, p.o.) > Silymarin (50 mg/kg, p.o.) > AQTB (400 mg/kg, p.o.).

Phytoconstituents like phenolic compounds and tannins were reported for their hepatoprotective and antioxidant effect (Fattah et al., 2005 and Kinjo. 2005) and these two were present in alcoholic and aqueous extracts. These active principles can be accounted for hepatoprotective effect. Further work is in progress to isolate and characterized the active principle in the extract.

It can be concluded that *Terminalia belerica* fruit extracts possess a protective effect against ethanol-induced hepatotoxicity in rats, as evidenced by the physical, biochemical and histological parameters.

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