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Antioxidant and Hepatoprotective Activity of Ethanolic Extracts of Bark of *Zanthoxylum armatum* DC in Paracetamol-Induced Hepatotoxicity

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ABSTRACT
The scientific evaluation of medicinal plants used in the preparation of folk remedies has provided modern medicine with effective pharmaceuticals for the treatment of diseases. The ethanolic extract of *Zanthoxylum armatum DC* bark was investigated for its hepatoprotective and antioxidant effects on Paracetamol (500 mg/kg) induced acute liver damage in Wistar albino rats. Hepatoprotection activity was measured by using biochemical parameters such as serum glutamate oxalate transaminase and se-rum Glutamate Pyruvate Transaminase (SGOT and SGPT), alkaline phosphatase (ALP), bilirubin and total protein. The ethanolic extract of *Zanthoxylum armatum DC*(EZA) at the doses of (100 mg/kg ,200 mg/Kg and 400 mg/Kg) produced significant hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin and lipid peroxidation while it significantly increased the levels of glutathione (GSH), catalyse (CAT) and super oxide dismutase (SOD) in a dose dependant manner. The effects of EZA were comparable to that of standard drug silymarin. These results suggest that EZA may have potential therapeutic value in the treatment of some liver disorders, probably by its antioxidative effect on hepatocytes.

Keywords: *Zanthoxylum armatum* DC, Hepatoprotective effect, Antioxidants, Paracetamol, Silymarin.

INTRODUCTION

A phytotherapeutic approach to modern drug development can provide many invaluable drugs from traditional system of medicinal plants. It has been suggested that plant derived antioxidants play a critical role in treating acute, subacute and chronic liver damage. [1] Liver injury induced by various hepatotoxins has been recognized as major toxicological problems for years.. Hepatic injury is associated with distortion of these metabolic functions. [2] Additionally, it is the key organ of metabolism and excretion is continuously and variedly exposed to xenobiotics, drugs, viral infections. If during all such exposures to the above mentioned challenges the natural protective mechanisms of the liver are overpowered, the result is hepatic injury.

In spite of growth of modern medicine, there are no single synthetic drugs available for the treatment of hepatic disorders. However there are several herbs/herbal formulations claimed have possess beneficial activity in treating hepatic disorders.

Zanthoxylum armatum DC. (Fam. Rutaceae), commonly known as tajbal (Hindi); an evergreen or sub-deciduous shrub, found in the hot valleys of the Himalayas from Jammu to Khasia hills at 600-1800 m and eastern ghats in Orissa and Andhra Pradesh at 1200 m, also sometimes planted for hedges in Assam. It is officially listed in the Ayurvedic pharmacopoeia of India, its important formulations are: Pancatikta, uggulu, ghot, kjlaka. [3] According to many Ayurvedic or traditional sources this plant is widely used as hepatic tonic for many liver problems. [4] On the other hand some of its scientifically proved applications are Anthelmintics [5], Antiviral Inhibition of keratinocyte growth [6], mosquito repellent etc. Chemically, the presence of Berberine, dictamnine, xanthoplanine, armatamid , asarinin and fargesin, alpha- and beta-amyrins, lupeol have been reported. [7-8] Recently, hepatoprotective activity of ethanolic extracts of bark evaluted CCl_4 induced hepatic damage. [9] However there is no scientific basis or reports in the modern literature regarding its usefulness effect as hepatoprotective agent against Paracetamol-induced hepatotoxicity. Paracetamol is one of the most widely used analgesics with few side effects when taken in therapeutic doses [10-11] and hepatotoxicity is a common consequence of Paracetamol overdose. [12] Thus the present study was conducted to evaluate its hepatoprotective potential against Paracetamol-induced hepatotoxicity in rats.

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MATERIALS AND METHODS

Chemicals

Paracetamol was purchased from National Chemical Pvt. Ltd, Baroda. All the routine reagents, routine chemicals were obtained from Ranbaxy chemicals Ltd, Delhi; Qualigens chemicals Ltd, Bombay; S.D. Fine chemicals, Boisar, Silymarin was provided by Admac Pharma Ltd, Panchkula, Berberine chloride was purchased from MP Biomedicals, Ohio. Thiobarbituric Acid (Spectrochem Pvt. Ltd), digonostic kits (Lab Care Diagnostics Pvt. Ltd),

Preparation of plant extract

Bark of *Zanthoxylum armatum* DC. was purchased from LVG Ayurvedic shop and authenticated with help of botanist at university. Bark was air dried for a week at 35-40^{o}C and pulverized in electric grinder. The powder obtained was extracted in ethanol.

Phytochemical studies

Qualitification of extract was evaluated by physical and chemical profile. Physical profile was done by evaluating total ash value, Acid insoluble ash, foreign matter, ethanol extractives, and water extractive parameter. [2]

Animals

All experiments and protocol described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Nirma University of Science and Technology, Ahmedabad with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The protocol no. was IPS/PCOL/MPH07/004. Healthy adult male rats (200-250 gms) were used for the study. Rats were housed in small cages with free access to food and water *ad libitum*. During the period of experiment the animals were fed with the standard laboratory diet.

Acute toxicity studies

The acute toxicity study for ethanolic extract of *Zanthoxylum armatum* DC Bark was performed using rats. The animals were fasted overnight prior to the experiment and maintained under standard conditions. The extracts were administrated orally in increasing dose of 2000 mg/kg of extract.

Induction of hepatic injury [13]

Experimental design

Six groups each comprising of six Wistar albino rats weighing in the range of 150-180 g was selected.

1. Normal Control (NC) : They were administered with vehicle (saline) for 28 days. Paracetamol was not given
2. Paracetamol Control group (PC) : Paracetamol (500mg/kg) was administered for 28 days orally.
3. Ethanolic extract of *Zanthoxylum armatum* DC (EZA100) : Ethanolic extract of *Zanthoxylum armatum* DC. (100 mg/kg p.o) was administered for 28 days along with paracetamol (500mg/kg p.o)
4. Ethanolic extract of *Zanthoxylum armatum* DC (EZA200) : Ethanolic extract of *Zanthoxylum armatum* DC. (200 mg/kg p.o) was administered for 28 day along with paracetamol (500mg/kg p.o)
5. Ethanolic extract of *Zanthoxylum armatum* DC (EZA400) : Ethanolic extract of *Zanthoxylum armatum* DC. (400 mg/kg p.o) was administered for 28 day along with paracetamol (500mg/kg p.o)
6. Silymarin 25mg/kg + Paracetamol (SILYMAR.) : Silymarin (25mg/kg p.o) was administered for 28 day along with paracetamol (500mg/kg p.o)

Animals were sacrificed 24 h after the last treatment. Blood was collected, allowed to clot and serum was separated at 2500 rpm for 15 min and biochemical investigations were carried out. Liver was dissected out and used for histopathological studies and antioxidant activity. [14]

Biochemical Studies

The estimation of various biochemical parameters namely SGOT, SGPT, ALP, serum bilirubin and Total protein were measured using an assay kit (Lab Care Diagnostics (India) Pvt. Ltd). Liver homogenates (5% w/v) were prepared in cold 50mM potassium phosphate buffer (pH 7.4) using a Remi homogenizer. The unbroken cells and cell debris were removed by centrifugation at 1000 rpm for 10 using a Remi refrigerated centrifuge. The supernatant was used for the estimation of reduced glutathione [15], malondialdehyde (MDA) [16], superoxide dismutase (SOD) [17] and catalase [18] levels.

Histopathology

From all the animals used for curative study small portion of liver tissue was fixed in 10% formaline saline, processed and embedded in paraffin wax to obtain 5-6µm thick hematoxylin and eosin stained sections

Statistical analysis

The data are expressed as mean±S.E.M. The difference among means has been analyzed by one-way ANOVA. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Phytochemical analysis

Phytochemical study Extract subjected for phytochemical study showed the presence of alkaloids, carbohydrates, proteins, amino acids, phenolic compounds, glycosides and flavonoids and results of physical profile were found within limit (Table 1 & Table 2).

Acute toxicity studies

Ethanolic and aqueous extracts did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose.

Effects of extracts on AST, ALT, ALP, total bilirubin and total protein

The results of hepatoprotective effect of extracts on Paracetamol- intoxicated rats are shown in (Table 2). In the Paracetamol- intoxication in normal rats elevated the levels SGPT, SGOT, ALP, total bilirubin and direct bilirubin were observed significantly indicating acute hepatocellular damage and billiary obstruction. The rats that received EZA showed a significant ($P<0.001$) decrease in all the SGOT, SGPT, ALP, total bilirubin and direct bilirubin, compared to induced control group.. So, the ethanol extract treated group (400mg/kg) was superior to the other extracts but not as effective as the silymarin.

Effects of extracts on MDA, GSH, SOD, CAT levels

Results are cited in Table 4 clearly revealed increase in the levels of MDA and hydroperoxides in Paracetamol-intoxicated rats compare to control group. Treatment with extracts significantly prevented this raise in levels. GSH, SOD and CAT content have significantly increased in extract treated groups whereas CCl_4-intoxicated group has shown

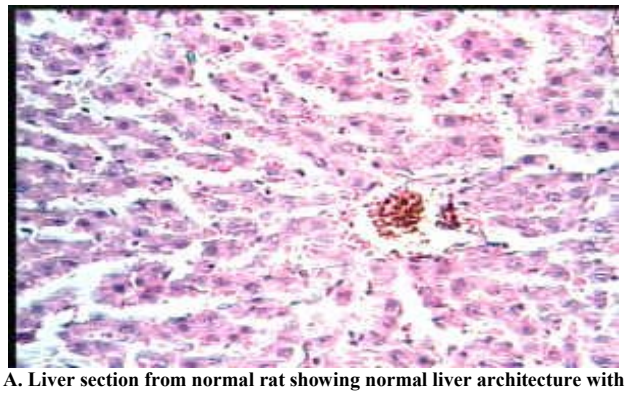
significant decrease in levels compare to control group. Ethanolic extract (400mg/kg) has shown maximum protection.

Table 1: Observation table of Physical Profile

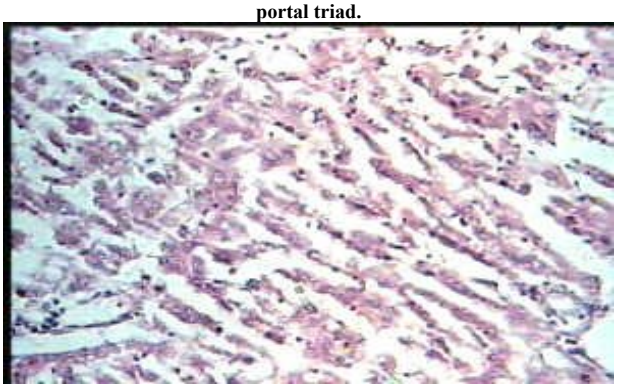
S. No	Evaluation Parameter	Yield in (%)	Limit
1	Foreign Matter	0.2 %	Not more than 2 %
2	Total ash	2.0%	Not more than12 %
3	Acid insoluble ash	0.5%	Not more than 1.5%
4	Ethanol Extractives	10.2%	Not less than 8.5 %
5	Water Extractive	16.1%	Not less than 13 %

Table 2: Observation table of Chemical test

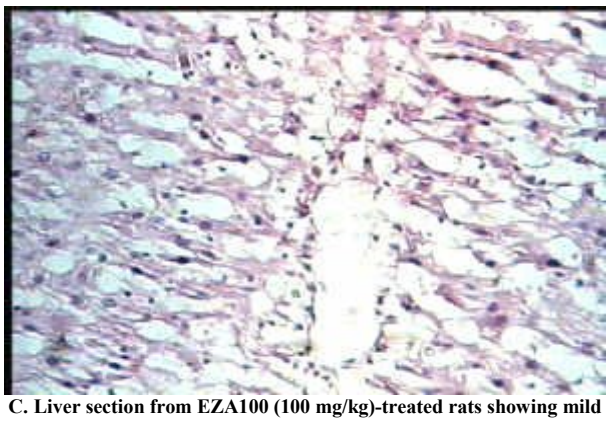
S. No	Constituents	Tests	Present{+}/ Absent (-)
1.	Alkaloids	Mayer's test	+
		Dragordraff's test	+
		Hager's test	+
		Wagner's test	+
2.	Sterols	Libermann's Burchard test	+
		Salkowski's –	+
3.	Carbohydrates And Glycoisdes	Molisch's test	+
		Fehling's test	+
		Benedict's test	+
		Borntrager's test	+
4.	Fixed Oils and Fats	Spot test	+
		Saponification test	+
5.	Phenolic Compound	$FeCl_3$ test	-
6.	Protein and Amino acids	Biuret test	-
		Ninhydrin test	-
		Xanthoprotein test	-
		Millon's test	-
7.	Triterpinoid and Saponins	Foam test	-
		Haemolysis test	-
8.	Tannins	Gelatin test	-
		$FeCl_3$ test	-
9.	Gums and Mucilage	Precipitation with 90% alcohol	-
10.	Flavonoids	Aqueous NaOH	+
		Conc. H_2SO_4	+



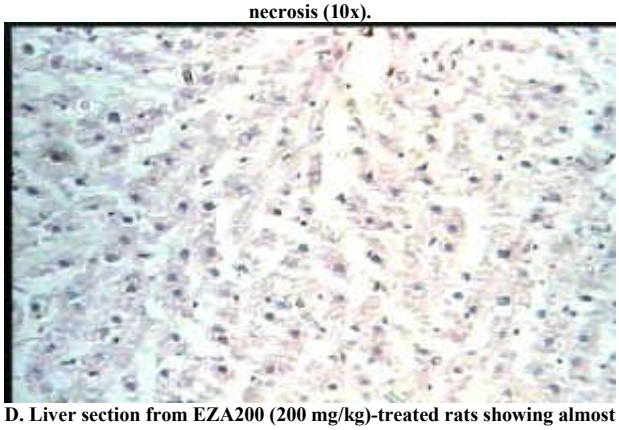
A. Liver section from normal rat showing normal liver architecture with portal triad.



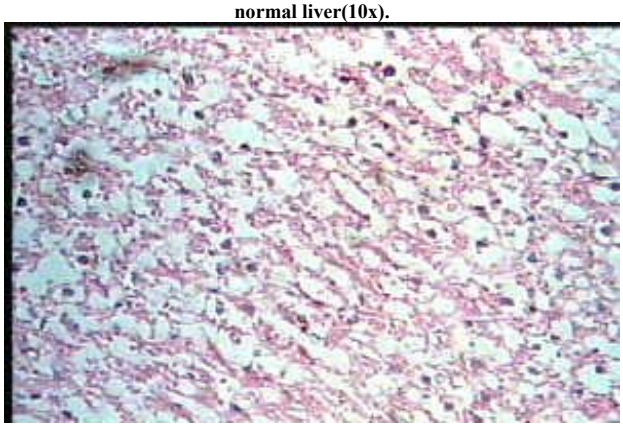
B. Liver showing severe degree of cell swelling, vacuolation and necrosis in rats treated with CCl_4 (10x).



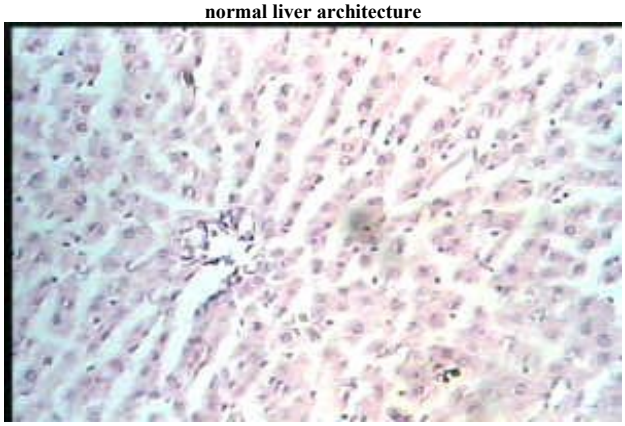
C. Liver section from EZA100 (100 mg/kg)-treated rats showing mild necrosis (10x).



D. Liver section from EZA200 (200 mg/kg)-treated rats showing almost normal liver(10x).



E. Liver section from EZA400 (400 mg/kg)-treated rats showing almost normal liver architecture



F. Liver section from EZA400 (400 mg/kg)-treated rats showing normal liver architecture

Fig. 1. Photomicrographs of liver sections of rats stained with haematoxylin and eosin (10x)

Table 3: Effects of Extract on antioxidant enzyme activity in Paracetamol induced Hepatotoxicity

Design of Treatment	SGOT (U/I)	SGPT (U/I)	ALP (U/I)	D.BIL. (mg/dl)	T. BIL (mg/dl)	T. Protein (mg/dl)
NC	70.42 ± 2.50	40.44± 1.05	89.76± 3.45	0.36± 0.01	1.33±0.07	7.98±0.088
PC (500 mg/kg, p.o)	170.17±2.10*	144.918 ± 3.46*	228.48±6.42*	0.97±0.03*	4.00±0.04*	5.27±0.053*
EZA-100 (100 mg/kg, p.o)	120.765± 2.36#	109.12 ± 2.97#	134.18± 5.36#	0.77± 0.01#	2.37±0.06#	6.06 ±0.032#
EZA-200 (200 mg/kg, p.o)	96.612 ± 2.10#	68.09 ± 1.67#	104.72± 4.82#	0.67± 0.02#	2.00 ±0.04#	6.89 ±0.14#
EZA-400 (400 mg/kg, p.o)	89.919 ± 2.5#	57.21 ± 1.54#	102.45± 3.04#	0.58± 0.01#	1.65± 0.03#	7.4 ± 0.05#
Silymarin (25 mg/kg, p.o)	85.26 ± 1.65#	52.11 ± 1.35#	99.28± 2.68#	0.57± 0.009#	1.56± 0.01#	7.53 ± 0.03#

Table 4: Effects of Extract on antioxidant enzyme activity in Paracetamol induced Hepatotoxicity

Design of Treatment	MDA (nmol/mg protein)	SOD (µ/mg protein)	Reduced glutathione (µg/ mg protein)	Catalase (U/mg protein)
NC	3.51± 0.37	7.21±0.29	15.94±0.96	22.08± 1.77
PC (500 mg/kg, p.o)	10.02±0.72*	3.58±0.40*	4.82±0.47*	10.92±0.52*
EZA-100 (100 mg/kg, p.o)	7.16± 0.44#	4.19±0.27#	9.78± 0.69#	13.91±0.42#
EZA-200 (200 mg/kg, p.o)	5.35± 0.41#	4.89±0.24#	11.83± 0.73#	15.45±0.61#
EZA-400 (400 mg/kg, p.o)	4.21± 0.37#	6.06 ± 0.35#	14.98± 0.59#	20.66±0.77#
Silymarin (25 mg/kg, p.o)	3.83± 0.33#	6.96 ± 0.24#	15.79± 0.29#	21.66± 0.74#

Histopathological observations

The normal architecture of liver (Fig. 1A) was completely lost in rats treated with Paracetamol (Fig. 1B) with the appearance of vacuolated hepatocytes and degenerated nuclei. Vacuolization, fatty changes and necrosis of hepatocytes were severe in the centrilobular region and these changes were also observed in areas other than the centrilobular regions.

The livers of rats treated with extract at a dose of 400 mg/kg (Fig.1E), 200 mg/kg (Fig. 1D) or silymarin 25 mg/kg (Fig. 1F) showed a significant recovery from Paracetamol induced liver damage as evident from normal hepatocytes with well defined nuclei. Vacuolization and fatty degeneration were remarkably prevented by the treatment with extracts and silymarin.

DISCUSSION

According to many Ayurvedic or tradional sources *Zanthoxylum armatum* plant is widely used as hepatic tonic for many liver problems. It is reported that of ethanolic extracts of bark have hepatoprotective activity against CCl4 induced hepatic damage. [9] However there is no scientific basis or reports in the modern literature regarding its usefulness effect as hepatoprotective agent against Paracetamol-induced hepatotoxicity. Paracetamol (acetaminophen), a widely used antipyretic and analgesic drug produces acute liver damage if accidental overdoses are consumed. The covalent binding of N acetyl-p-benzoquinoneimine, an oxidation product of paracetamol, to sulphydryl groups of protein results in cell necrosis and lipid peroxidation. [19] This induces a decrease in glutathione levels, which is attributed to be the cause of hepatotoxicity, have, as reported earlier. [20]

In the assessment of liver damage by paracetamol the determination of enzyme levels such as SGPT and SGOT is largely used. Necrosis or membrane damage of hepatocytes, releases the enzyme into circulation; therefore, it can be measured in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. Serum ALP and bilirubin level on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure. [21]

This present study evaluated the hepatoprotective effects of Ethanolic extract of *Zanthoxylum armatum* (EZA) in paracetamol induced liver toxicity. Acute administration of paracetamol produced a marked elevation of the serum levels of SGOT, SGPT, ALP, serum bilirubin and total proteins in treated animals (Group II to VI) when compared with that of the normal control group (Group I). Treatment with EZA at a dose of 100 mg/kg, 200 mg/kg and 400mg/kg significantly reduced the elevated levels of these enzymes EZA dose 400 mg/kg significantly reduced the elevated enzyme levels that were comparable to the standard drug silymarin. Lipid peroxidation has been postulated as being the destructive process in liver injury due to paracetamol administration. [22] In our study, elevations in the levels of TBARS in liver of rats treated with paracetamol were observed. The increase in TBARS levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with EZA significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of ethanolic extract of *Zanthoxylum armatum* is due to its antioxidant effect

To sum up the above discussion, altogether it is proved that the ethanolic extract of *Zanthoxylum armatum* DC. has significant beneficial action of Ethanolic extract of *Zanthoxylum armatum* (EZA) in paracetamol induced liver toxicity.. Our findings support the reported therapeutic use of this herb in in paracetamol induced liver toxicity as a hepatoprotective agent in Indian system of medicine

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