Research Paper

Evaluation of Hepatoprotective Activity of *Zanthoxylum armatum* on Paracetamol-induced Liver Toxicity in Rats

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The present study was carried out to identify phytochemical compounds and to study the hepatoprotective activity of *Zanthoxylum armatum* rhizome. Phytochemical analysis was carried out using standard procedures to quantify total alkaloid and phenolic contents. Hepatoprotective activity was determined using paracetamol-induced hepatotoxicity in rats. *Zanthoxylum armatum* extracts showed the presence of steroids, terpenoids, flavonoids, alkaloids, glycosides, phenols, oils, tannins and carbohydrates. The methanol extract has more phenolic and alkaloid contents than other extracts. The methanol extract at 500 mg/kg showed greater hepatoprotective activity with 66.87, 64.84, 67.95, 60.76 and 65.85 % protection on aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin and total protein enzyme levels of the liver, respectively. The results of the present study and previous reports indicated that a variety of phytochemical constituents in *Zanthoxylum armatum* and its' extracts contributed to the observed antioxidant, antibacterial and hepatoprotective activities.

Key words: Paracetamol, phytochemical compounds, rhizome, *Zanthoxylum armatum*

The liver plays a vital role in physiological functions that include oxidation, reduction, hydrolysis, conjugations, sulfation and acetylation in detoxification along with carbohydrate, lipid and protein metabolism of the body[1,2]. If it did not function normally to regulate body metabolism, which reflects in alterations in different enzymes, hormones and proteins levels[3,4]. This will affect the health conditions of the human or any living organism leading to mortality[5]. So, it is very important to maintain a healthy liver to have a healthy body with effectively functioning organs. Nowadays, a variety of factors cause injury to different organs of the body including liver and these include microbial infections, environmental pollutants and toxic chemicals. In recent studies, hepatotoxic chemical or drugs are found to cause more liver diseases compared to spontaneous liver diseases. Simultaneously, oxidative stress also plays an important role in damaging liver tissue and it is one of the major causes for liver damage around the world[5-8]. Many drugs used to treat different diseases produce therapeutic benefit but also simultaneously cause side effects that at times lead to different organ damage in the body[3,9]. Antibacterial drugs on long-term usage become ineffective due to microorganisms acquiring resistant to these drugs[10]. The antibiotic-resistant microorganisms are a huge threat to global human health[11]. In this regard, identification of new drugs from various sources is very important to control the increasing disease burden including liver diseases and side effects of existing drugs[12].

Identification of new active metabolites from medicinal plants has been proved to be the best approach since olden days mainly since herbal medicine has been the basis for current day drugs[13-15]. Indian Ayurveda, Chinese traditional medicine, Traditional Korean medicine, Unani medicine, Traditional African medicine were mainly based on medicinal plants[16-19]. In recent studies, various medicinal plants were reported to possess biological activities, bioactive metabolites, precursors for of new bioactive molecules and therapeutic potential against several diseases including hepatic injury[20-22]. However, many more medicinal plants still need to be investigated for potential biological activities that these might possess.

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Zanthoxylum armatum is one of the traditional medicinal plants used as an aromatic tonic in fever and dyspepsia, toothache, rheumatism and as a lotion for scabies while, fruits of this plant were used in the preparation of essential oils[23,24]. Because of its aromatic nature, it is also used as a deodorant and disinfectant. Some early reports on *Z. armatum* indicated that the leaves exhibited anticonvulsant and antispasmodic effects[25,26], while the stem bark was cytotoxic and antioxidant[27]. However, there have been no published reports on the hepatoprotective ability and phytochemical composition of roots of *Z. armatum* except that a previous study from our laboratory reported antioxidant activity[28].

MATERIALS AND METHODS

The solvents used in the present study were of analytical grade and the diagnostic kits for estimation of different enzymes levels were acquired from Span Diagnostics Ltd., Gujarat, India. The standard drug (Liv 52) and paracetamol were purchased from a local medical store, Visakhapatnam, India. Liv 52 is a multiherbal drug from Himalaya Global Holdings, which is used to treat chemically-induced hepatotoxicity.

Preparation of extracts:

The *Z. armatum* plant material was collected in the Tirupathi region and authenticated in the Department of Botany, Sri Venkateswara University, Tirupathi. The collected material was washed under running water and dried under shade. The dried plant material was made into coarse powder. The powdered material was extracted separately with ethyl acetate, chloroform and methanol using Soxhlet extraction procedure. The collected solutions were concentrated on a rotavap to obtain dry extracts, which were stored in desiccator and used for further studies.

Phytochemical analysis:

The collected extracts were tested for the presence or absence of different biologically active compounds and quantified the total phenolic, alkaloid contents using standard test procedures[2,29-32].

Experimental animals:

To evaluate the hepatoprotective activity of *Z. armatum*, outbred Wistar albino rats (180-250 g) were used, which were obtained from M/s Mahaveer Enterprises, Hyderabad, India and the work was carried at College of Pharmaceutical Sciences, Andhra University, approved Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Reg. No. 516/01/A/CPCSEA). Animals were maintained at controlled environmental conditions before and during the experiments (22±2°, 60±5% humidity). Animals were provided with standard laboratory diet and water.

Acute toxicity study:

The extracts of *Z. armatum* were tested for their toxicity as per Organisation for Economic Co-operation and Development test guideline 423[33]. Wistar rats of single sex (male; n=6) were grouped into three. After one week of maintenance at laboratory conditions, each group of animals were administered orally 2000 mg/kg of a test extract and the animals were kept under observation to monitor mortality, physiological and psychological condition such as skin changes, morbidity, aggressiveness, oral secretions, sensitivity to sound and pain and respiratory movements.

Hepatoprotective activity of *Z. aramatum*:

The ethyl acetate extract of *Z. armatum* (ZAEAE), chloroform extract (ZACE) and methanol extract (ZAME) were tested in the paracetamol-induced liver toxicity model[34]. The selected animals for the study were assigned to XII groups (n=6), group I and II animals were administrated with normal saline for 7 d (2 ml/kg), group III was administrated with Liv 52 (25 mg/kg), groups IV to VI were administrated with 125, 250 and 500 mg/kg of ZAEAE, groups VII to VIII were administrated with 125, 250 and 500 mg/kg of ZACE and groups X to XII were administrated with 125, 250 and 500 mg/kg of ZAME extract for 7 d. On the 5th d of experiment excluding group I, all were treated with 200 mg/kg paracetamol. On the end of the experiment (day 7), 2 h after the administration of the final dose of test extracts, blood samples were collected through retro-orbital plexus from all animals under light anaesthesia. The collected samples were immediately centrifuged (2400 rpm/ 15 min) and the serum was separated. Serum was used to estimate liver functional parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and total protein content using an Autoanalyzer[2] (RM4000, Biochemical systems International, Italy).

RESULTS AND DISCUSSION

Among different extracts of the selected plant, ZAME

showed consistent and better activity compared to other extracts. So, based on these results and availability of extract, ZAME was used for isolation of pure compounds through column chromatography. Column chromatography was carried out using 10 g of extract with 60-120 mesh silica gel as absorbent. The column was eluted in a gradient approach with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol mixtures from 100 % hexane to 100 % methanol with increasing polarity. A mixture of white crystals was isolated at 10 % hexane in ethyl acetate.

Now a days, the identification of new biological activities and isolation, characterization of new molecules from medicinal plants became the main focus of many research studies[35,36]. Several reports of various bioactive molecules from natural resources including medicinal plants that have been indicated in the traditional systems of medicine, with a potential to be useful in the treatment of many diseases have appeared in the literature[37-40]. Hence the current research work was carried out and the qualitative phytochemical screening of *Z. armatum* extracts revealed the presence of different phytochemical constituents like steroids, terpenoids, flavonoids, alkaloids, glycosides, phenols, oils, tannins and carbohydrates. The extracts gave negative results for the presence of amino acids and quinones. The ZAME revealed the presence of oils but ZACE and ZAEAE gave negative results. The ZAME showed more phenolic content 23.79±0.17 (gallic acid equivalents) and alkaloid content 29.44± 0.82 (mg/g) than other extracts. The results are presented in Tables 1 and 2.

TABLE 1: PHYTOCONSTITUENTS IN DIFFERENT EXTRACTS OF *Z. ARMATUM*

Phytochemicals	Extracts of *Z. armatum*		
	Chloroform	Ethyl acetate	Methanol
Phytosterols	+	+	+
Terpenoids	+	+	+
Glycosides	+	+	+
Saponins	-	+	+
Flavonoids	+	+	+
Tannins	-	+	+
Carbohydrates	-	+	+
Alkaloids	+	+	+
Amino acids	-	-	-
Oils	-	-	+
Quinones	-	-	-
Phenols	+	+	+

'+' denotes presence and '-' denotes absence

TABLE 2: TOTAL PHENOLIC AND ALKALOID CONTENTS OF *Z. ARMATUM* EXTRACTS

Name of the extract	Total phenolic content (GAE)	Total alkaloidal content (mg/g)
Ethyl acetate	19.48±0.84	23.92±0.76
Chloroform	20.37±0.22	19.77±0.18
Methanol	23.79±0.17	29.44±0.82

n=3; mean±SEM

The *Z. armatum* root extracts were tested for toxicity in rats at a dose of 2000 mg/kg, which revealed no mortality or any behavioural changes. The hepatoprotective activity of *Z. armatum* extracts was evaluated in the paracetamol-induced liver toxicity model in rats at different concentrations (125, 250 and 500 mg/kg) selected on the basis of the toxicity studies in which 2000 mg/kg dose of these extracts was found to be safe. The hepatoprotective activity was evaluated by the ability of the extract to produce significant changes in the levels of the liver biomarkers such as AST, ALT, ALP, total bilirubin and total protein that were altered by treatment with paracetamol. Percent protection was calculated as follows, % protection = (AST/ALT/ALP/ total bilirubin in toxicant group)–(AST/ALT/ALP/total bilirubin in drug group+paracetamol group)/(AST/ ALT/ALP/total bilirubin in the paracetamol group)– (AST/ALT/ALP/total bilirubin before treatment)×100.

Group I rats treated with vehicle showed no significant changes in the biomarkers of liver function while group II rats treated with paracetamol showed significant changes in these biomarkers (Table 3). The group III rats administered with paracetamol (200 mg/kg, subcutaneous) and Liv 52 (25 mg/kg per day, per os) showed significant changes in liver biomarker levels compared to group I and II and the percent protection produced by Liv 52 in AST, ALT, ALP, total bilirubin and total protein levels were, 94.79, 92.97, 96.49, 97.57 and 90.24 %, respectively ($p<0.01$).

Group IV, V and VI rats were treated with ZAEAE extract at 125, 250 and 500 mg/kg and the percent protection in AST, ALT, ALP, total bilirubin and total protein levels offered by these treatments was 13.24, 10.78, 10.54, 9.55% and 9.76, 27.57, 26.88, 27.33, 23.44 % and 27.44, 52.95, 52.19, 53.42, 45.14 and 52.44 %, respectively ($p<0.01$).

Rats of the groups VII, VIII and IX were treated with ZACE extract at 125, 250 and 500 mg/kg and the treatments offered percent protection for AST, ALT, ALP, total bilirubin and total protein levels to the tune

TABLE 3: BIOMARKER LEVELS IN GROUPS I TO XII AFTER VARIOUS TREATMENTS

Drug treatment (mg/kg)	Enzymes				
	AST (U/l)	ALT (U/l)	ALP (U/l)	Total bilirubin (mg/dl)	Total protein (g/dl)
Control	89.17±1.11	52.33±1.73	183.50±0.85	0.25±0.01	6.93±0.06
Paracetamol	332.17±2.18	159.0±0.89	529.83±3.04	2.17±0.06	4.20±0.04
Liv 52 (25)	101.83±1.72	59.83±1.11	195.67±1.56	0.29±0.01	6.67±0.08
ZAEAE (125)	300.00±1.29	147.50±1.09	493.33±1.15	1.98±0.08	4.47±0.08
ZAEAE (250)	265.17±1.35	130.33±2.20	435.17±1.42	1.72±0.04	4.95±0.07
ZAEAE (500)	203.50±3.57	103.33±1.74	344.83±2.34	1.30±0.04	5.63±0.11
ZACE (125)	307.50±2.23	146.17±1.49	471.83±2.14	1.93±0.07	4.60±0.05
ZACE (250)	268.50±1.98	132.83±1.40	415.83±1.96	1.73±0.07	4.97±0.10
ZACE (500)	208.67±2.06	105.17±1.92	306.00±2.48	1.33±0.07	5.70±0.09
ZAME (125)	277.50±2.05	138.67±0.67	451.33±2.25	1.80±0.05	4.80±0.05
ZAME (250)	226.00±1.65	114.33±1.61	368.50±1.82	1.40±0.05	5.43±0.06
ZAME (500)	169.67±2.16	89.83±2.01	294.50±1.67	1.00±0.07	6.00±0.07

n=6 and mean±SD

of 10.15, 12.03, 16.75, 12.15 % and 14.63, 26.20, 24.53, 32.92 22.57 % and 28.05, 50.82, 50.47, 64.63, 43.40 and 54.88 %, respectively ($p<0.01$).

Rats of the groups X, XI and XII were treated with ZAME extract at 125, 250 and 500 mg/kg doses and the treatments showed percent protection of AST, ALT, ALP, total bilirubin and total protein levels to the extent of 22.50, 19.06, 22.67, 19.10 % and 21.95, 43.69, 41.88, 46.58, 39.93 % and 45.12, 66.87, 64.84, 67.95, 60.76 and 65.85 %, respectively. These results were shown in Tables 3, and 4 ($p<0.01$).

Isolated compound ZA-1 was obtained as a white powder with melting point of 210° and was identified as a compound with formula $C_{29}H_{48}O$ and *m/z* 412.37 [M+H] (fig. 1). The IR spectrum has shown a broad bands at 3321.54, 2923.43, 2852.95, 1459.55 and 1036.52 cm^{-1} indicating the presence of a hydroxyl and a double bond in the molecule based on ^{1}H NMR, ^{13}C NMR, ESI-MS, FT-IR and ν_{max} spectral data of the compound (fig. 2). The mass spectrum exhibited a molecular ion [M]+ peak at *m/z* 426 from EI-MS. The ^{13}C NMR data revealed 30 carbon signals where seven methyls, ten methylene, six methine carbons, five quaternary carbons and two olefinic carbons. The olefinic methylene protons are seen as singlets at 4.68 and 4.55 ppm. The ^{13}C NMR data was also in complete agreement with the existence of an isopropenyl group, in particular, the characteristic vinylic carbon atom resonances at 151.2 and 109.3 ppm, corresponding to carbon atoms 20 and 29, respectively (fig. 3A). This supported the olefinic methylene protons seen as

TABLE 4: ^{1}H (400 MHZ) ^{13}CNMR (100 MHZ) SPECTRAL DATA OF LUPEOL IN $CDCL_3$

Position	δH	δC
1	2.41 1.03	38.7
2	2.40 4.68 (s, 1H, 1OH)	27.2 78.7
3	-	38.8
4	0.73	55.2
5	2.39	18.3
6	2.38 1.92	34.2
7	-	40.8
8	1.91(dd, 2H)	50.4
9	-	37.1
10	2.73	20.9
11	1.91 3.14	25.2
12	1.90 (t, 3H)	
13	3.13	38.7
14	-	43.0
15	3.14 1.89 (m, 3H)	27.7
16	2.47(m, 1H)	35.5
17	-	43.2
18	1.93	48.4
19	4.55 (ddd,1H)	48.3
20	-	
21	3.15 (m, 1H) 1.91	151.2
22	1.93(m, 2H) 1.26	29.8
23	0.79 (s, 3H)	40.2
24	0.94 (s,3H)	28.0
25	0.74 (s,3H)	15.4
26	0.93 (s, 3H)	16.1
27	1.89 (s, 3H)	16.0
28	1.85 (s, 2H) 0.82 (s, 3H)	14.5 18.0

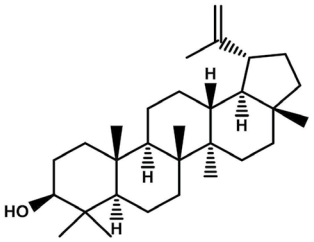

Fig. 1: Structure of lupeol

singlets at 4.68 and 4.55 ppm in the ^{1}H NMR spectrum was found to be consistent with known compound lupeol (figs. 3B and Table 4).

The *Z. armatum* extracts of rhizomes showed concentration-dependent hepatoprotective activity by restoring the altered liver biomarker levels due to the hepatic injury caused by paracetamol to normal levels.

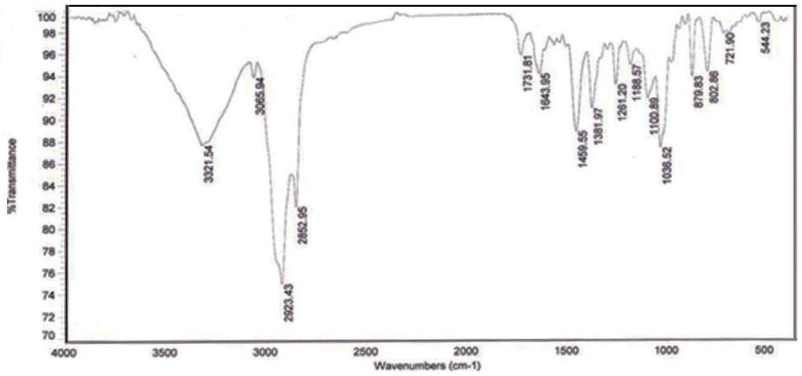

Fig. 2: FTIR spectrum of the compound ZA-1, lupeol

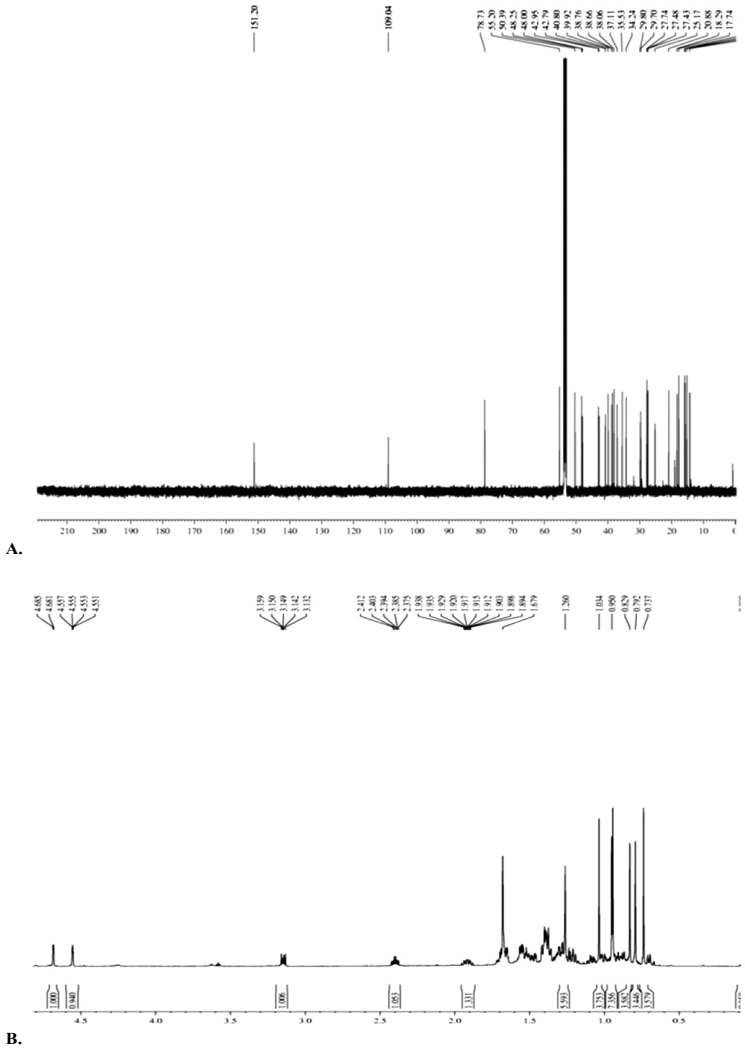

Fig. 3: (A) ^{13}C NMR, and (B) ^{1}H NMR spectrum of the compound ZA-1, lupeol

TABLE 5: PERCENT PROTECTION OFFERED BY VARIOUS DOSES OF EXTRACTS OF *Z. ARMATUM* AGAINST PARACETAMOL-INDUCED LIVER TOXICITY

Name of the enzyme	Dose of extract									Liv 52
	125 mg/kg			250 mg/kg			500 mg/kg			
	Extract type			Extract type			Extract type			
	Ethyl acetate	Chloroform	Methanol	Ethyl acetate	Chloroform	Methanol	Ethyl acetate	Chloroform	Methanol	
AST (U/l)	13.24	10.15	22.50	27.57	26.20	43.69	52.95	50.82	66.87	94.79
ALT (U/l)	10.78	12.03	19.06	26.88	24.53	41.88	52.19	50.47	64.84	92.97
ALP (U/l)	10.54	16.75	22.67	27.33	32.92	46.58	53.42	64.63	67.95	96.49
Total bilirubin (mg/dl)	9.55	12.15	19.10	23.44	22.57	39.93	45.14	43.40	60.76	97.57
Total protein (g/dl)	9.76	14.63	21.95	27.44	28.05	45.12	52.44	54.88	65.85	90.24

Paracetamol is a widely used medication for fever and pain because of its low cost and effectiveness, but its overdose and long-term usage cause adverse effects that include liver damage. The ZAME showed better hepatoprotection compared to the other two extracts in the present study. These extracts showed variations in the percent protection offered for on individual biomarkers, but all extracts showed better protection of ALP levels and lesser protection of total bilirubin levels (Table 3) and as the concentration of extracts increased, the percent protection was also increased (Table 5). Recently it was observed that drug-induced liver toxicity is one of the main causes of mortality[41] and the mechanism of drug-induced liver damage was unclear. Some recent reports indicated that the liver damage is more due to free radical formation induced by excessive use of hepatotoxic drugs. These drugs affect organs functionality, enhance their metabolism leading to free radical formation and breakdown of macro molecules[42]. Enhanced production of free radicals, which are more unstable molecules, cause break down of cell membranes of cells including liver cells and alter molecular stabilization[43]. In previous reports, *Z. armatum* extracts demonstrated better protective nature through reducing different free radicals, which was found to be more pronounced with the ZAME[28]. Lupeol the compound isolated from this plant extract was previously reported to possess different pharmacological activities like antiinflammatory, antiprotozoal, antimicrobial. Qualitative analysis of *Z. armatum* extracts in this investigation revealed the presence of phenolics, alkaloids, terpenoids and glycosides, which were reported to exert a variety of biological activities[44-48]. Attempts to isolated pure components from the ZAME resulted in the isolation of lupeol, which was biologically active[49]. Phenolic compounds, alkaloids and terpenoids could also have been responsible for the observed hepatoprotective activity as the ZAME had more phenolic and alkaloid content and also simultaneously exerted greater antioxidant and hepatoprotective activities. Previous studies reported that extracts with more phenolic contents possessed more antioxidant activity and hepatoprotective activity[50,51]. Results from the current study indicated that it is likely to develop new drugs from the chemical constituents of *Z. armatum* extracts provided the mechanism of action of the hepatoprotective effect exerted is elucidated.

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Conflicts of interest:

There is no conflict of interest.

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