IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

INVESTIGATION ON HEPATOPROTECTIVE ACTIVITY OF *ZIZYPHUS XYLOPYRUS (RETZ.)* WILLD STEM BARK EXTRACTS AGAINST CARBONTETRACHLORIDE INDUCES LIVER DAMAGE

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Abstract

In the present study, the hepatoprotective effects of petroleum ether and methanol extract of *zizyphus xylopyrus* stem bark extract were studied using the model of hepatotoxicity induced by carbon tetrachloride (CCl(4)) in rats. CCl(4) administration induced a significant decrease in serum total protein, albumin, urea and a significant increase (P <or= 0.01) in total bilirubin associated with a marked elevation in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as compared to control rats. Further, CCl(4) intoxication caused significant increase in the TBARS and decrease in glutathione (GSH) levels in serum, liver and kidney. Both the extracts resulted in significant decreases in the activities of AST, ALT and ALP, compared to CCl(4)-treated rats. The results indicate that *zizyphus xylopyrus* stem bark extract possesses potent hepatoprotective effects against CCl(4)-induced hepatic damage in rats.

1. INTRODUCTION

Liver disease refers to a group of disorders of the liver that can lead to decompensate liver function. The liver has multiple functions and is the principal detoxifying organ, acting in the clearance of pathogens, toxic chemicals and metabolic waste products from the body as well as the synthesis of many key enzymesthat regulate these metabolic processes, which leads to either an increase in free radicals or reactive oxygen species (ROS) generation and/or a decrease in theantioxidant defense mechanisms. Acute liver disease is defined as a rapid hepatic dysfunction that occurs in theabsence of previous history of chronic liver disease; it is caused, for example, by excessive consumption of antibiotics or acetaminophen. By contrast, chronic liver disease is a long-term dynamic process that involves persistent hepatocytic destruction and regeneration. Major risk factors for chronic liver disease are hepatitisB viral and hepatitis C viral infection and alcoholic

liver-induced injury leading to alcoholic liver disease (ALD) as well as a group of metabolic disorders that can leadto nonalcoholic fatty liver disease (NAFLD).

2. Material and Methods

2.1 Collection and authentication of plant materials

Stem bark of *Z. xylopyrus* (Retz) Willd was collected from Bhainsa, Sagar (M.P.). The plants specimen was authenticated (Bot./H/02/49/02) by Dr. Pradeep Tiwari, Department of Botany, Dr. Hari Singh Gour Central University, Sagar (M.P.),

2.2 Extractions of plant material

500 gm of stem bark powder was packed in Soxhlet apparatus and extracted with methanol. For fractionation, dried methanolic extract (ZXME) was dissolved in water and then fractionated with petroleum ether (40-60ºC.) and ethyl acetate, to yield petroleum ether fraction (ZXPEF), ethyl acetate fraction (ZXEAF) and aqueous fractions (ZXAF).

2.3 Qualitative Chemical Analysis

Qualitative chemical analysis was performed on different plant extracts/fractions for the identification of various phytoconstituents (Harborne, 1984; Evans, 1996; Kokate *et al*., 2003).

2.4 Quantitative Estimation of Phytoconstitutents

Determination of Total Phenolics Content (TPC)Principle

The total phenolic content (TPC) was determined by the reported Folin-Ciocalteu method**Determination of Total Flavonoid Content**

Determination of total flavonoid content (TFC) is based on measurement of the intensity of red colour complex formed due to reaction between flavonoids and aluminum trichloride ($AlCl_3$) **(Olajuyigbe and Afolayan, 2011)**.

2.5 Determination of Antioxidant activity

2.5.1 DPPH radical scavenging

The DPPH radical scavenging ability was calculated by using the following equation

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

2.5.2 Hydroxyl radical scavenging assay

The OH° radical scavenging ability was calculated by using the following equation

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

2.5.3 Inhibition of lipid peroxidation in rat liver homogenatePrinciple

Percent inhibition of LPO was calculated using following equation.

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

2.6 Hepatoprotective activity of ZX extracts/fractions

Selection of animals

In the present investigation the Swiss albino rats of either sex, weighing between 180-240g were used. The animals were procured from College of Veterinary Sciences and Animal Husbandry, Mhow, (M.P.), India. Animals were allowed to acclimatize for two weeks before commencing the study and maintained under standard laboratory conditions (25±2 ºC temperature, 45-65% relative humidity and 12 h light and 12 h dark cycle). The animals were fedwith standard laboratory animal feed and water *ad libitum* throughout the study. The animal experimental protocol (AIPS/2018/2635/IAEC/04) was duly approved by the Institution Animal Ethical Committee (IAEC No.1546/PO/RE/S/11/CPCSEA).

Acute Toxicity Studies

Acute toxicity was determined following the acute toxicity class method (OECD guideline No. 420, 2001). The animals (non-pregnant female Wistar albino rats) were divided into different groups consisting three animals each. The all animals were fasted overnight with free access to water, weighed and a single dose (2000 mg/kg) of test substance was administered.

The normal control animals received a similar volume of 1% (*w/v*) aqueous carboxy methylcellulose (CMC) solution. Animals were observed individually during first 30 minutes, periodically during 48 hours with special attention given during first 4 hours (short-term toxicity)and daily thereafter for total of 14 days (short-term toxicity). The various sign and symptoms including tremors, convulsions, salivation, diarrhoea, sleep and coma were observed carefully. There was no lethality seen in any of the groups after 14 days of experiment and all the tested compounds were found to safe upto 2000 mg/kg. On the basis of these studies, following dose levels were selected for *in vivo* studies 100, 200 and 400 mg/kg.

Hepatoprotective effects against CCl_4 induced liver damage

Hepatoprotective are those therapeutic agents, which mitigate liver damage caused by hepatotoxic agents. The hepatoprotective activity of plant drugs and herbal formulations are studied against CCl_4 induced hepatotoxicity in experimental animals as they mimic any form of naturally occurring liver diseases. The experimental animals are usually treated with plant extractunder investigation for a specified period of time. The hepatotoxic agent is usually administered near the end of experimental period for induction of acute toxicity or in several doses during the course of experiment for chronic toxicity. Measuring the certain biochemical parameters and comparing their levels with normal, hepatotoxic and treated groups assess the activity of test material.

Experimental Preparation of Dosage Forms

Weight quantity of extract/fractions and silymarin (standard drug) (100 mg each) were triturated with 10 ml of 1% CMC solution in pestle-mortar continually for 15 min to gethomogenous suspension (100 mg/ml). All the suspensions were stored in air tight bottle in a cooldry place.

Preparation of Toxin Solutions

The CCl_4 was uniformly mixed with olive oil in the ratio of 1:1 (*v/v*) and this mixture wasinjected subcutaneously in rats at a dose level of 2 ml/kg,.

Experimental Design

The animals were divided into fourteen groups (n = 6) and initial body weight was recorded.

Group I (Normal control): Rats received vehicle (1% CMC solution) daily for 5 days and olive oil (1 ml/kg, s.c.) on days 2 and 3.

Group II (CCl_4 control): Rats received vehicle daily for 5 days and CCl_4: olive oil (1:1, 2 ml/kg, s.c.) on day 2 and 3.

Group III (Standard control): Received silymarin (50 mg/kg, b.w.) daily for 5 days and CCl_4: olive oil (1:1, 2 ml/kg, s.c.) on day 2 and 3.

Groups IV-VI (Treatment control): Received ZXEAF at a dose of 100, 200 and 400 mg/kg, b.w., respectively for 5 days and CCl_4: olive oil (1:1, 2 ml/kg, s.c.) on day2 and 3.

Groups VII-IX (Treatment control): Received ZXME at a dose of 100, 200 and 400 mg/kg, b.w., respectively for 5 days and CCl_4: olive oil (1:1, 2 ml/kg, s.c.) on day2 and 3.

Table 2.1: Dose regimen for CCl_4 induced hepatotoxicity

Group	Treatment	Duration in Days	Days of withdrawal of blood and liver
I	Vehicle(1% CMC solution	5	6^{th}
II	CCl_4 : Olive oil	5	6^{th}
III	Silymarin+ CCl_4	5	6^{th}
IV	ZXEAF(100mg/kg,) + CCl_4	5	6^{th}
V	ZXEAF(200mg/kg,) + CCl_4	5	6^{th}
VI	ZXEAF(400mg/kg,) + CCl_4	5	6^{th}
VII	ZXME(100mg/kg,) + CCl_4	5	6^{th}
VIII	ZXME(200mg/kg,) + CCl_4	5	6^{th}
IX	ZXME(400mg/kg,) + CCl_4	5	6^{th}

Estimation of Serum Parameters

The blood samples collected from rats were allowed to clot for 45 minutes at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 minutes and analyzed for various biochemical parameters: AST (SGOT), ALT (SGPT), ALP, LDH, and TB were determined following standard methods. The detailed procedure

is described in literatures available with kit which are as follows:

3. Results and Discussion

3.1 Result of Extraction of *Z. xylopyrus* stem bark

Table 3.1: Percentage yield of *Z. xylopyrus* stem bark extracts

S. No.	Extracts	Physical state	Yield (% *w/w*)
1	Methanolic extract (ME)	Semisolid	7.28%
2	Petroleum ether fraction (PEF)	Semisolid	1.28%
3	Ethylacetate fraction (EAF)	Semisolid	3.47%
4	Aqueous fraction (AF)	Solid	2.56%

3.2 Preliminary phytochemical screening (Qualitative chemical analysis)

Preliminary phytochemical analysis results of ZX extracts showed that ZXME contains carbohydrate, glycosides, flavonoids, tannins, phytosterols, triterpenoids, alkaloids, fixed oil and fats; ZXPEF contains phytosterols, triterpenoids, fixed oil and fats; ZXEAF contains glycosides, flavonoids, tannins while ZXAF contains carbohydrate, glycosides, flavonoids, tannins, alkaloids while protein and amino acids was found absent in all extracts.

Table 3.2: Phyotchemical screening of *Z. xylopyrus* stem bark extract/fractions

Tests for carbohydrate	ZXME	ZXPEF	ZXEAF	ZXAF
Molisch's test	+	-	-	+
Fehling's test	+	-	-	+
Benedict's test	+	-	-	+
Tests for alkaloids				
Mayer's test	+	-	-	+
Dragendorff's test	+	-	-	+
Hager's test	+	-	-	+
Wagner's test	+	-	-	+
Tests for glycosides				
Legal's test	+	-	+	+

Keller-Killiani test	+	-	+	+
Modified Borntrager's test	-	-	-	-
Tests for flavonoids				
Foam test	+	-	+	+
Alkaline Reagent Test	+	-	+	+
Shinoda's Test	+	-	+	+
Tests for phytosterols and triterpenoids				
Libermann's test	+	+	-	+
Libermann's burchard test	+	+	-	+
Salkowaski test	+	+	-	+
Tests for protein and amino acids				
Millon's test	-	-	-	-
Millon's reagent	-	-	-	-
Ninhydrin test	-	-	-	-
Tests for tannins				
Ferric chloride test	+	-	+	+
Lead Acetate test	+	-	+	+
Gelatin test	+	-	+	+
Test for fixed oils and fat				
Filter paper test	+	+	-	-
Saponification test	+	+	-	-

3.3 Quantitative estimation of phytoconstitutents

3.3.1 Determination of total flavonoids content (TFC) and total phenolic content (TPC)

Table 3.3: Amount of TPC and TFC present in ZX stem bark extract/fractions

Extract	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)
ZXME	107.29±0.36	40.46± 0.22
ZXPEF	18.24±0.27	0.48± 0.06
ZXEAF	174.78±0.78	50.64± 0.64
ZXAF	58.27±0.54	9.76± 0.73

Values are presented as mean±SEM; (n=3). GAE, Gallic acid equivalent; QE, Quercetinequivalent.

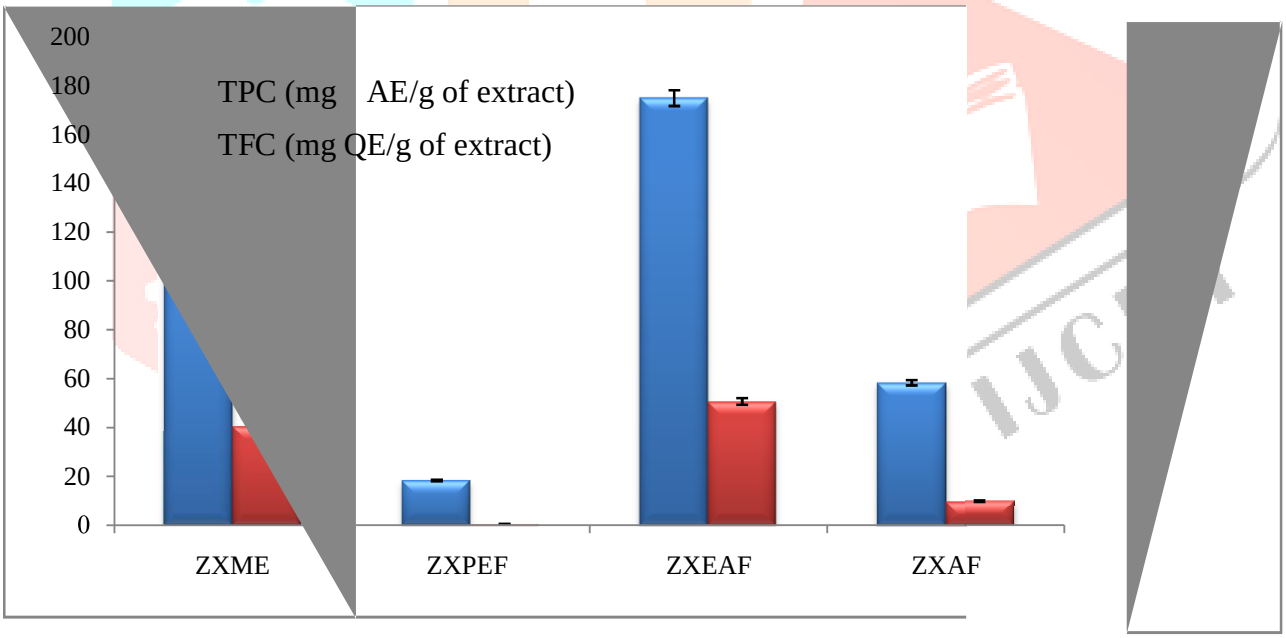


Fig 3.1: Amount of TFC and TPC present in ZX extracts/fractions

3.4 Determination of antioxidant activities

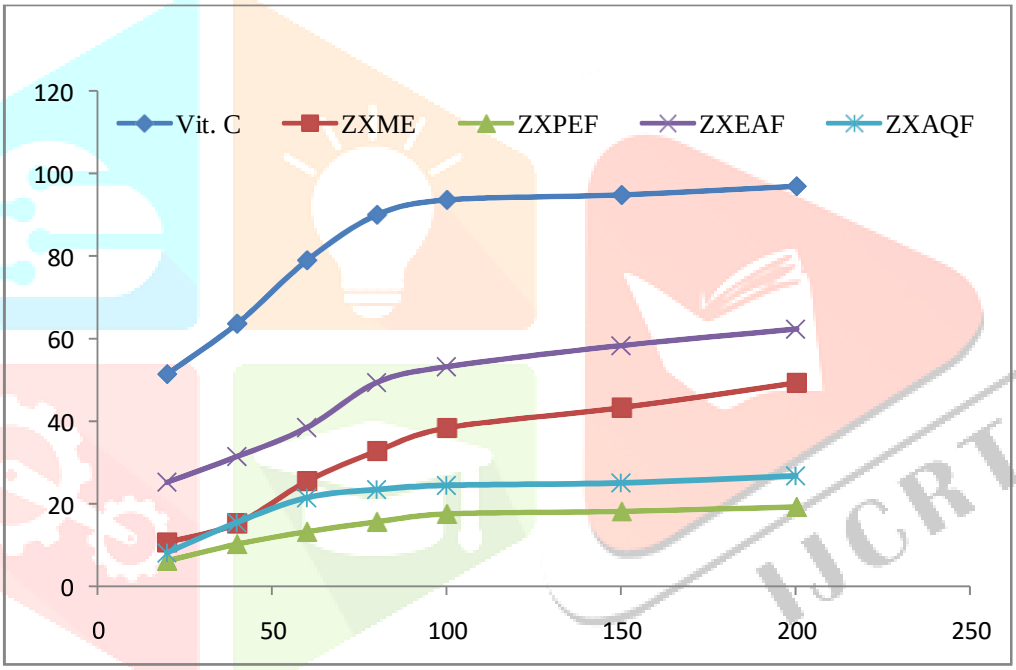
3.4 .1 DPPH radical scavenging assay

Table 3.4: Percent inhibition by ZX extract/fractions in DPPH radical scavenging assay

Con (µg/ml)	Vit. C	ZXME	ZXPEF	ZXEAF	ZXAQF
10	28.4±0.64	7.4±0.24	2.7±0.12	18.7±0.54	3.2±0.67
20	51.4±0.32	10.7±0.17	6.3±0.28	25.3±0.26	8.2±0.98
40	63.6±0.27	15.3±0.72	10.3±0.34	31.5±0.18	15.5±1.06

60	78.9±0.54	25.5±0.36	13.3±0.74	38.5±0.48	21.5±0.42
80	89.9±0.49	32.8±0.42	15.7±0.59	49.4±0.76	23.5±0.69
100	93.5±0.62	38.4±0.84	17.6±0.62	53.2±0.49	24.5±0.74
150	94.7±0.21	43.3±0.17	18.2±0.18	58.3±0.25	25.1±0.97
200	96.8±0.18	49.3±0.23	19.3±0.29	62.3±0.81	26.8±0.08
IC_{50}	**16.6±0.72**	**179.7±2.64**	**546.8±1.78**	**116.9±0.86**	**368.8±0.23**

Values are presented as mean±SEM; (n=3)



Percent inhibition by ZX extract/fractions in DPPH radical scavenging assay

3.4 .2 Hydroxyl radical (OH°) scavenging assay

Table 3.5: Percent inhibition by ZX extract/fractions in OH° scavenging assay

Con (µg/ml)	Vit. C	ZXME	ZXPEF	ZXEAF	ZXAQF
10	19.2±0.23	10.1±0.89	5.2±0.46	13.1±0.36	6.1±0.54
20	32.7±0.49	18.3±1.02	7.4±0.89	21.3±0.28	10.3±0.28
40	67.4±0.34	27.1±1.14	10.1±0.74	32.1±0.79	12.1±0.47
60	78.2±0.67	34.2±0.76	14.3±0.68	41.2±0.64	16.2±0.78
80	86.2±0.89	36.4±0.89	16.6±0.42	48.4±0.51	20.1±0.82
100	89.7±0.12	39.1±0.42	17.5±0.29	54.1±0.87	24.2±0.96
150	92.3±0.26	42.2±0.54	18.6±0.94	62.2±1.12	26.4±0.64
200	96.8±0.74	45.4±0.69	19.3±0.65	72.4±0.54	30.1±0.89
IC_{50}	**26.3±0.84**	**193.9±2.01**	**581.4±1.54**	**105.7±0.63**	**339.8±1.29**

Values are presented as mean±SEM; (n=3)

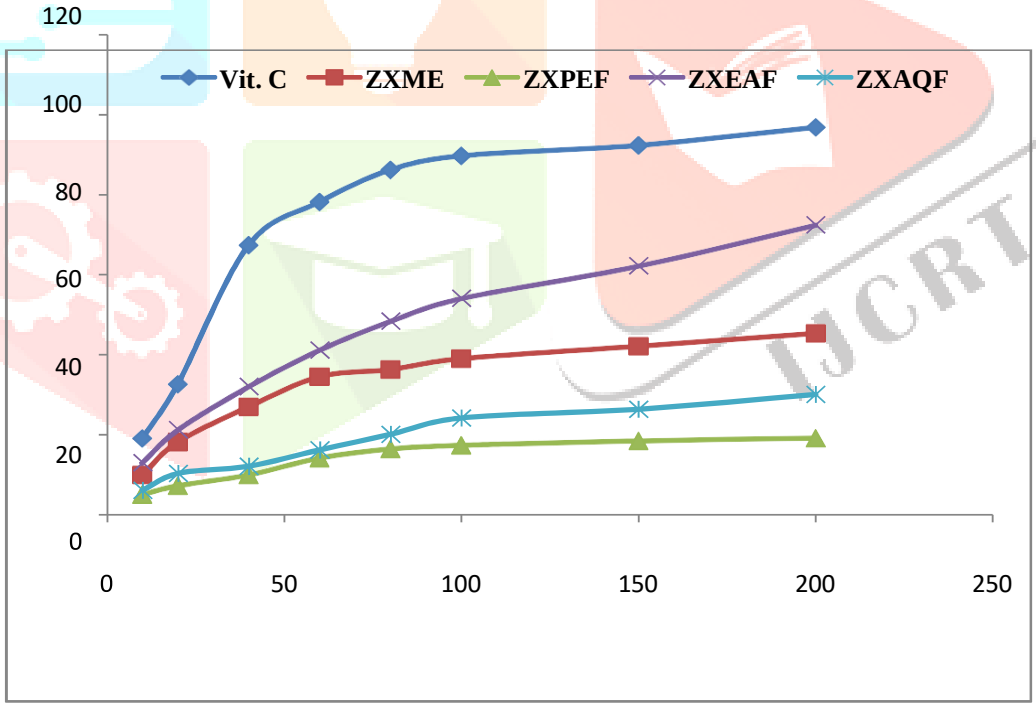


Fig. 7.5: Percent inhibition by ZX extract/fractions in OH° scavenging assay

3.4 .3 Inhibition of lipid peroxidation (LPO) in rat liver homogenate

Table 3.6: Percent inhibition by ZX extracts/fractions in inhibition of LPO assay

Con (µg/ml)	Vit. C	ZXME	ZXPEF	ZXEAF	ZXAQF
20	13.6±0.26	8.6±0.18	4.5±0.59	12.4±0.71	7.1±0.78
40	25.7±0.48	13.9±0.29	7.9±0.44	18.7±0.32	9.8±0.27
60	41.9±0.12	21.6±0.74	11.2±0.26	26.9±0.45	13.2±0.34
80	62.8±0.78	29.4±0.36	13.6±0.18	35.4±0.49	16.1±0.68
100	78.7±0.49	31.2±0.48	14.9±0.59	42.6±0.98	19.7±0.92
150	82.4±0.58	36.2±0.22	21.6±0.47	61.9±0.1.02	25.9±0.45
IC_{50}	73.6±0.79	196.2±3.84	372.2±2.89	119.3±1.96	311.3±2.81

Values are presented as mean ± SEM; (n=3)

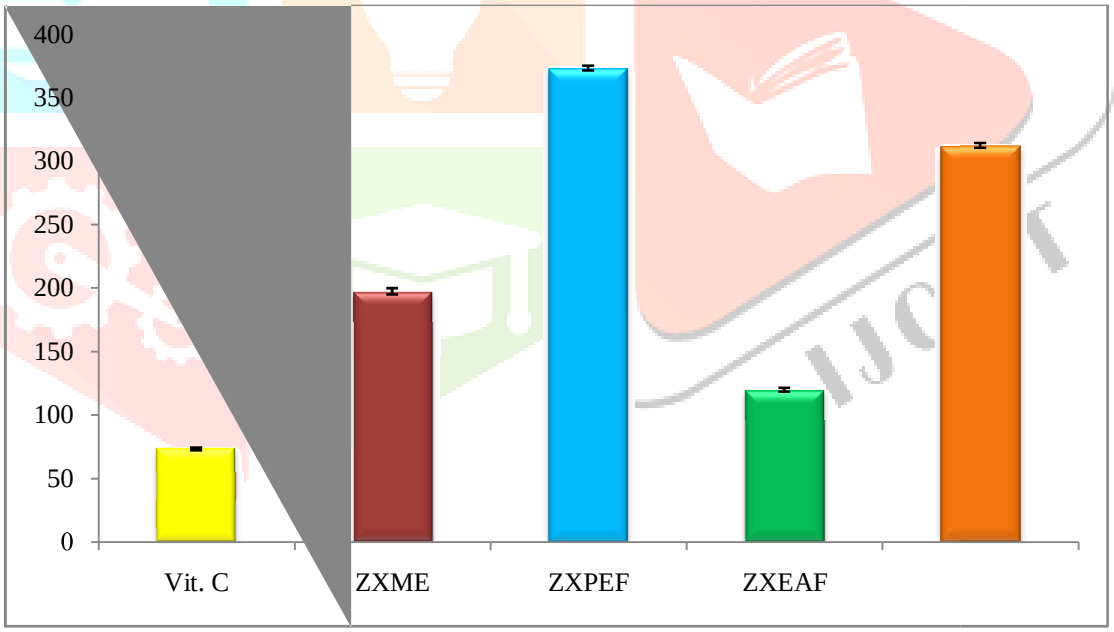


Fig. 7.6: Percent inhibition by ZX extract/fractions in inhibition of lipid peroxidation assay

Evaluation of *in vivo* hepatoprotective effects of ZXME and ZXEAF

3.5 Acute oral toxicity

No adverse changes and mortality were observed in animals, which orally receivedZXME and ZXEAF up to 2000 mg/kg of body weight. This indicates that 2000 mg/kg is maximum safe dose. So 1/20th, 1/10th and 1/5th *i.e.* 100, 200 and 400 mg/kg of body weight, of the maximum safe dose were selected for studying *in vivo* hepatoprotective effect.

Evaluation of *in vivo* hepatoprotective ZXME and ZXEAF against CCl_4 induced liverdamage

Various pharmacological and chemical substances that belong to intrinsic or idiosyncraticgroups of hepatotoxins may induce hepatic damage varying from asymptomatic hepatic functional disturbance to widespread liver necrosis **(Teocharis *et al*., 2001)**. In present study CCl_4 was used as an intrinsic hepatotoxin, to determine the protective effects of the active extracts of ZX i.e. ZXME and ZXEAF. Enzymatic activation of CCl_4 by cytochrome-P450 leads to the formation of CCl_3° free radical which interfere and combines with cellular protein and lipids; leading to cell necrosis. CCl_4 also interfere with transport function of liver cell whichleads to leakage of SGOT and SGPT from cell cytoplasm in to serum. It also impaired/damaged bile excretion, consequently serum ALP and bilirubin increase. CCl_4 also interfere with metabolism of cholesterol and triglyceride transportation leading to fatty liver. Administration of CCl_4 induced a significant ($p<0.001$) rise in serum enzymes levels which are in agreement with previous findings **(Jain *et al*., 2008)**.

In present study, substantial increase in serum enzyme activities was observed upon CCl_4 administration revealing its toxic effect on liver. The CCl_4 control group showed a significantrise in the serum levels of SGOT (3.62 fold), SGPT (5.92 fold), ALP (3.58 fold) and TB (3.32 fold). In this group, the elevated levels were found to be 185.3±4.23, 158±7.09, 123.43±2.78 and2.26±0.32, respectively as compared to normal group in which the levels were 51.17±5.47, 26.65±2.23, 34.45±1.89 and 0.68±0.08, respectively.

Hepatoprotective effects of ZXME against CCl_4 induced liver damage:

The effect of ZXME on CCl_4-induced hepatotoxicity is presented in **table.** Treatment of rats with ZXME (100, 200 & 400 mg/kg) caused moderate reduction in the elevated levels of serum marker enzymes when compared to CCl_4 control group. At 100 mg/kg, ZXAF showed percent protection of 23.41%, 18.64%, 12.64% and 16.23%, respectively for the levels of serum SGOT, SGPT, ALP and TB. At 200 mg/kg it reduced the same levels by 36.82%,32.46%, 23.73% and 28.73%, respectively. However with higher dose (400 mg/kg) it significantly reduced the elevated levels by 42.93%, 42.73%, 34.80% and 39.15%, respectively. In this group, the decreased levels were found to be SGOT (127.76±4.83), SGPT (101.89±3.46),6ALP (92.46±4.85), and TB (1.64±0.18), respectively. However, treatment with silymarin (50 mg/kg) also exhibited a significant reduction in the raised levels of SGOT (89.13±8.11), SGPT (58.3±3.20), ALP (65.87±1.73) and TB (1.19±0.09) as compared to ethanol control group and showed percent protection of 71.72%, 75.90%, 64.68%, and 67.72%, respectively.

The histological observations also showed very less hepatoprotective effect of ZXME. Atlower doses (100 & 200 mg/kg) ZXME treated rats shows completely damaged central lobular vein, degenerative changes and mild necrosis in this group**.** However, at higher dose (400 mg/kg) centrilobular vein still damage, wider sinusoids were clearly visible. Necrosis and degenerative changes were noticeably decreased as compared to low dose still fatty changes and hypertrophy in hepatocytes were seen. Although, at this dose, the protective effect of ZXAF were negligible compared to that silymarin.

Table 3.7: Effects of ZXEAF and silymarin (SIL) on SGOT, SGPT, ALP and TB in CCl_4- induced hepatotoxicity in rats

Biochemical Parameter	Normal	CCl_4:Olive oil (1:1,2)	Sil (50mg/kg +CCl_4)	ZXEAF (100mg/kg +CCl_4)	ZXEAF (200 mg/kg + CCl_4)	ZXEAF (400mg/ kg +CCl_4)
SGOT (IU/L)	51.17	185.38	89.13	131.47	113.39	96.89
SGPT (IU/L)	26.65	158.03	58.3	114.95	92.46	62.88
ALP (IU/L)	34.45	123.43	65.87	104.38	89.38	81.41
TB (mg/dl)	068	2.26	1.19	1.76	1.42	1.11

Each values represents the mean±SEM; (n=6), *p<0.05, **p<0.01, ***p< 0.001 respectively when compared with toxicant control group (CCl_4) (one- way ANOVA followed by Dunnett's test).Values in parentheses indicate percent hepatoprotective activity (H), calculated as 100 x (value of CCl_4 control – value of treatment) / (value of CCl_4 control – value of normal control).

Table 3.8: Effects of ZXME and silymarin (SIL) on SGOT, SGPT, ALP and TB in CCl_4- induced hepatotoxicity in rats

Biochemical Parameter	Normal	CCl_4:Olive oil (1:1,2)	Sil (50mg/kg +CCl_4)	ZXME (100mg/kg +CCl_4)	ZXME (200 mg/kg + CCl_4)	ZXME (400mg/ kg +CCl_4)
SGOT (IU/L)	51.17	185.38	89.13	153.96	135.96	127.76
SGPT (IU/L)	26.65	158.03	58.3	133.54	115.38	101.89
ALP (IU/L)	34.45	123.43	65.87	112.18	102.32	92.46
TB (mg/dl)	068	2.26	1.19	2.03	1.81	1.64

Each values represents the mean±SEM; (n=6), *p<0.05, **p<0.01, ***p< 0.001 respectively when compared with toxicant control group (CCl_4) (one- way ANOVA followed by Dunnett's test).Values in parentheses indicate percent hepatoprotective activity (H), calculated as 100 x (value of CCl_4 control – value of treatment) / (value of CCl_4 control – value of normal control).

Conclusion

Based on the phytochemical screening, *in vitro* antioxidant and *in vivo* screening studies, it is finally concluded that ethylacetate fraction (ZXEAF) of *Z. xylopyrus* stem bark have noticeable hepatoprotective activities in the tested models.Results also suggested that the activities may be due to antioxidant property of the extracts; which is attributed due to presence of phenolics and flavonoids along with other phytoconstitutents. However, these effects may also partly be due to other phytoconstitutents present in these extracts. Based on present study, these plant extracts can be used efficiently as hepatoprotective in case of acute hepatic injury. The experimental evidences related to hepatoprotective effects proved the usefulness of

Z. xylopyrus stem bark in the treatment of acute hepatic disorders. Thefurther studies needed to isolate and characterize the phytoconstitutents and their detailed mechanism/s of action responsible for hepatoprotective effects of *Z. xylopyrus* stem bark extract.

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