ORIGINAL ARTICLE

Phytochemical analysis, antioxidant activity, and hepatoprotective effects of *Zizyphus xylopyrus* (Retz.) Willd leaves extracts against carbon tetrachloride-induced hepatotoxicity in *in vitro* and *in vivo* models

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Abstract

Introduction: This work was done to determine phytochemical content, antioxidant activity, and hepatoprotective effects of *Zizyphus xylopyrus* leaves extracts against carbon tetrachloride-induced hepatotoxicity in *in vitro* and *in vivo* models. **Materials and Methods:** The total flavonoids content (TFC), total phenolic content (TPC), and total tannin content (TTC) were determined using quercetin and tannic acid equivalents, as standard while antioxidant activities of extracts were determined using the standard *in vitro* methods. All the extracts subjected to *in-vitro* HepG2 cell line study as well as to evaluate *in vivo* hepatoprotective effects against carbon tetrachloride (CCl_4) intoxicated rats. **Results:** Among all extracts, ethyl acetate extract (EAE) possess potent antioxidant activity, viz., ferric reducing ability of plasma (abs = 0.379 ± 0.07), 2,2-diphenyl-1-picrylhydrazyl (inhibitory concentration 50% [IC_{50}]: 103.50 ± 2.05 µg/mL), $OH^{\bullet}$ (89.33 ± 1.79 µg/mL), $NO^{\bullet}$ (IC_{50} 129.34 ± 1.29 µg/mL), O_2^{-} (IC_{50} 62.03 ± 2.78 µg/mL), and inhibition of lipid peroxidation (110.05 ± 2.96 µg/mL). Treatment with EAE significantly increased the cell viability (IC_{50} 80.93 ± 1.02 µg/mL) by preventing CCl_4 induced cell damage in *in-vitro* HepG2 cell line. In case of both prophylactic and curative study, EAE extract significantly ($P < 0.001$) decreased CCl_4-induced increased serum liver enzymes activities in CCl_4-intoxicated rats, comparable to silymarin. Hepatoprotective potential further supported by pentobarbitone induced sleeping time and improved hepatic tissue histopathology. Study results suggest that antioxidant activity and hepatoprotective effect of EAE might be due to presence of polyphehols, *viz.,* TFC (43.76 ± 0.78 Quercetin equivalent [QE] mg/g extract), TPC (194.16 ± 0.74 gallic acid equivalent [GAE] mg/g extract), and TTC (20.45 ± 2.31 GAE mg/g extract). Reversed-phase high-performance liquid chromatography analysis results showed highest quercetin content (32.8 ± 0.24 mg/g) in EAE. **Conclusion:** This study advocated that due to the presence of flavonoids, *Z. xylopyrus* leaves exhibited marked antioxidant and hepatoprotective activities.

Key words: Antioxidant, flavonoids, free radicals, hepatoprotective effect, *Zizyphus xylopyrus*

INTRODUCTION

The liver plays a pivotal role in the metabolism and is continuously exposed to xenobiotics, environmental pollutants and chemotherapeutic agents, which leads to either an increase in free radicals or reactive oxygen species (ROS) generation and/or a decrease in the antioxidant defense mechanisms/or directly affect the biochemistry

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of cell by interacting with cellular macromolecules.[1,2] Oxidative stress and inflammation are well-noted features in the pathogenesis of hepatic disorders. The percentage of liver toxicity due to various toxicants exposures is much higher in developing countries like India (8-30%) as compared to the advanced countries (2-3%). Approximately, 20000 deaths and 250000 new cases have been reported every year worldwide.[3,4] The available synthetic drugs such as interferon and corticosteroids for treatment of hepatic disease are expensive and may cause further damage.

Physicians and patients are in need of effective therapeutic agents with a low incidence of side-effects.[5] Therefore, considerable attention is focused on sighting the hepatoprotective agents from plants based medicines, which can attenuate the free radicals resulting in the reduction of the oxidative damage to the biomolecules. Various reports suggested that phytochemicals such as phenolics, alkaloids, terpenoids, isoterpenoids, and quinines possessed protective effect in free radical associated disorders.[6,7]

Natural polyphenols such as flavonoids (e.g., Quercetin, kaempferol, and rutin) and other phenolic acids (hydroxyl cinnamic acid derivatives, gallic acid, and catechins) are potent antioxidants exhibiting hepatoprotective effect and are used in the treatment of chronic liver injuries.[8,9] Carbon tetrachloride (CCl_4) is one of the hazardous chemicals, which undergoes enzymatic activation by various cytochromes such as CYP2E1, CYP2B1 or CYP2B2 and possibly CYP3A, for generating the trichloromethyl radical ($CCl_3^{\bullet}$), which binds to the cellular molecules (i.e., Nucleic acid, protein, and lipid) and impairs the crucial cellular processes such as lipid metabolism (fatty degeneration-steatosis). This radical reacts with molecular oxygen to form trichloromethylperoxy radical ($CCl_3OO^{\bullet}$) and initiates the lipid peroxidation (LPO), which affects the permeability of mitochondria, endoplasmic reticulum and plasma membrane, resulting in the loss of cellular calcium sequestration, homeostasis and subsequent cell damage.[10,11]

The genus *Ziziphus* (Rhamnaceae) comprises about 100 species of evergreen tree known for its various pharmacological activities, i.e., hypoglycemic, hypotensive, antimicrobial, antioxidant, immunostimulant, anti-inflammatory, and hepatoprotective activity.[12] *Ziziphus xylopyrus* (Retz.) Willd, a large, straggling shrub, is commonly distributed throughout the North-Western India, Pakistan, and China. Various reports claimed the traditional uses of the different parts of the plant for the treatment of different ailments such as obesity, urinary troubles, diabetes, skin infections, fever, diarrhea, insomnia, digestive, and liver disorders.[13] Different types of the plant extracts have been advocated pharmacologically to exhibit the antisteroidogenic,[14] anticonvulsant, antinociceptive, anti-inflammatory,[15] antidepressant[16] as well as wound healing activity.[17-19]

Functional foods are foods thought to supply an additional benefit beyond meeting micronutrient or macronutrient needs, such as reducing oxidative stress, improving longevity, or lowering some disease risk factor.[8] *Z. xylopyrus* leaves were reported to contain flavonoids, *viz.,* Quercetin and querceitrin,[20] which can ameliorate oxidative stress-mediated liver damage. It is also used in Aragvadhadi Kvath Churna and Abharak Bhashma formulations.[21,22] The link between antioxidant and hepatoprotective mechanisms has been previously established.[23] Therefore, various parts of *Z. xylopyrus* exhibit potential to be included as active component of functional food.[24] However, till date, no attempt has been made to determine the phytochemical contents, antioxidant activity, and hepatoprotective potential of *Z. xylopyrus* leaves. In this regard, this study was aimed to determine the antioxidant activity as well as pharmacological evaluation of the protective and curative effect of different extracts of *Z. xylopyrus* leaves against CCl_4 induced hepatic injury.

MATERIALS AND METHODS

Chemicals and Reagents

Quercetin, gallic acid, vitamin C, trichloroacetic acid, thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), sodium nitroprusside, hydroxylamine hydrochloride, penicillin, and Streptomycin were purchased from Sigma-Aldrich, Mumbai. The CCl_4, Folin–Ciocalteu reagents (FCR), 1-napthylethylenediamine dihydrochloride, sulfanilamide, and ortho phosphoric acid were purchased from SD Fines Chemicals Pvt. Ltd., Mumbai. Trypsin, Dulbecoo's Modified Eagle's Medium (DMEM), dimethylsulfoxide (DMSO), Trypan blue, ethylenediaminetetraacetic acid (EDTA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Bioworld, Mumbai and Hi-Media, Bengaluru, respectively. The Silymarin was received from Micro Lab, Baddi as gift sample. All other chemicals and solvents of analytical grade used in this study were procured from local supplier.

Plant Material Collection, Authentication, and Extraction

Fresh leaves of *Z. xylopyrus* were collected from plants growing in Talchini, Sagar (Madhya Pradesh), India in June 2012. The plant specimen was authenticated by Prof. Pradeep Tiwari, Department of Botany, Dr. H. S. Gour Central University, Sagar (Madhya Pradesh), India. A voucher specimen (Her/Bot/02/49/04) was prepared and deposited in the herbarium of the department. Dried powdered leaves of *Z. xylopyrus* were extracted successively with petroleum ether (40-60°C), chloroform, ethyl acetate and ethanol by hot extraction process. The marc was suspended in distilled water, macerated for 48 h and filtered. All the filtrates were dried under vacuum using rotary evaporator (Superfit Pvt Ltd., Mumbai, India) at 40 ± 2°C to obtained petroleum ether extract (PEE) (5.18% w/w), chloroform extract (CHE)

(2.0% w/w), ethyl acetate extract (EAE) (1.69% w/w), ethanol extract (EtOHE) (7.98% w/w), and aqueous extract (AQE) (6.23% w/w).

Determination of Phytoconstituents

Preliminary phytochemical screening

The PEE, CHE, EAE, EtOH, and AQE extracts of *Z. xylopyrus* were subjected to determine the presence of various phytoconstituents such as alkaloids, glycosides, flavonoids, tannins, phytosterols, carbohydrate, and amino acids using previously reported qualitative chemical tests.[25]

Total flavonoid content (TFC)

Determination of TFC was based on measurement of the intensity of red color complex formed due to reaction between flavonoids and aluminum trichloride ($AlCl_3$).[26] Briefly, the $AlCl_3$-methanolic solution (1 mL, 2% w/v) was mixed to various diluted extracts or standard (1 mL) and allowed to stand for 1 h at 25 ± 2°C before the absorbance was measured at λ_{max} 420 nm against blank using ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu 1800, Kyoto, Japan). Extract samples were evaluated at a final concentration of 1 mg/mL. The TFC was compared to quercetin equivalent (QE) (mg/g of extract) using the regression equation y = 0.00036x + 0.01768, r^2 = 0.9798, where x is the absorbance and y is the QE.

Total phenolic content (TPC) and total tannin content (TTC)

The TPC was determined by the reported Folin–Ciocalteu method, while TTC was estimated by precipitating tannins with gelatin.[27] Briefly, various extracts (1 mL, 1% w/v) were mixed with FCR (10 mL, previously diluted with distilled water in 1:10 ratio), vortexed and set aside for 5 min, then sodium carbonate solution (10 mL, 7% w/v) was added and diluted up to 25 mL with distilled water. The mixture was allowed to stand for 1.5 h at 25 ± 2°C and absorbance was measured at λ_{max} 765 nm against blank using UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan).

For the estimation of non-tannin phenolics, the extract (2 mL) was mixed with gelatin solution (100 mg/mL of distilled water) and allowed to stand for 15 min at 4°C, vortexed and filtered through Whatman filter paper no.1. The filtrate (0.5 mL) was diluted with distilled water (up to 1 mL) and non-tannin phenolics were estimated by the similar method as used for TPC. The TTC of extracts was determined by subtracting non-tannin phenolics from the TPC. The results were compared to gallic acid equivalent (GAE) (mg/g of extract) using the regression equation y = 0.01695x + 0.14004, r^2 = 0.9895, where x is the absorbance and y is the GAE.

Determination of Antioxidant Activity

Ferric reducing ability of plasma (FRAP) assay

The ferric ion reducing power of extracts was determined by measuring the absorbance of chromophore.[28] Different concentrations of extracts or standard, vitamin C (1 mL, 20-200 µg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6), potassium ferric cyanide (2.5 mL, 1% w/v) and incubated at 50 ± 2°C for 20 min. Trichloroacetic acid (2.5 mL, 10% w/v) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was separated and mixed with 2.5 mL of distilled water and ferric chloride (0.5 mL, 0.1% w/v). Then, the absorbance was measured at λ_{max} 700 nm using UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan).

DPPH radical scavenging assay

The DPPH° radical scavenging ability of extracts was determined by measuring the intensity of yellow colored complex formed due to reaction between proton donar and DPPH° radical.[29] In short, the methanolic solution of DPPH (3 mL, 0.01 mM) was mixed with extracts or vitamin C (3 mL, 10-200 µg/mL) and incubated for 30 min in the dark. The absorbance of DPPH solution was measured at λ_{max} 517 nm using UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan). The DPPH° radical scavenging ability was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (A_0 - A_t / A_0) \times 100 \qquad \text{(Equation 1)}$$

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

Hydroxyl radical scavenging assay

Hydroxyl radicals ($OH^{\bullet}$) scavenging ability of the extracts was determined by measuring the intensity of hydroxylated salicylate complex formed by the reaction of $OH^{\bullet}$ radical and sodium salicylate.[26] The reaction mixture containing ferrous sulfate (1 mL, 1.5 mM), hydrogen peroxide (H_2O_2) (0.7 mL, 6 mM), sodium salicylate (0.3 mL, 20 mM), and varying concentrations of extracts or vitamin C (1 mL, 10-200 µg/mL) were incubated for 1 h at 37 ± 2°C. After incubation absorbance of the reaction mixture was measured at λ_{max} 562 nm using UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan). The $OH^{\bullet}$ radical scavenging ability was calculated using the Equation 1.

Nitric oxide ($NO^{\bullet}$) radical scavenging assay

The determination of $NO^{\bullet}$ scavenging ability of the extracts is based on the inhibition of $NO^{\bullet}$ radical generated from sodium nitroprusside in phosphate buffer saline solution by Griess reagent (1% sulfanilamide, 2% orthophosphoric acid, and 0.1% naphthyl ethylenediamine dihydrochloride).[30] Briefly, sodium nitroprusside (0.6 mL, 5 mM) solution was mixed with and without varying the concentration of the extracts

or vitamin C (2 mL, 10-200 µg/mL) and incubated at 25 ± 2°C for 5 h. Incubated solution (2 mL) was mixed with equal volume of Griess reagent and absorbance of the purple colored azo dye chromophore was measured at λ_{max} 546 nm using UV-Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan). The NO• radical scavenging ability was calculated using the Equation 1.

Superoxide radical scavenging assay

Superoxide radical (O_2^-) scavenging activity of extracts was measured by reported NBT reagent method. This method is based on the generation of O_2^- by auto-oxidation of hydroxylamine hydrochloride in the presence of NBT, which gets reduced to nitrite. The nitrite ion in the presence of EDTA produced a color that was measured at λ_{max} 560 nm.[31] Sodium carbonate (1 mL, 50 mM), NBT (0.4 mL, 24 mM), and EDTA (0.2 mL, 0.1 mM) solutions were added in the test samples of extracts or vitamin C (1 ml, 10-200 µg/mL) and immediately absorbance was measured at λ_{max} 560 nm. The addition of hydroxylamine hydrochloride (0.4 mL, 1 mM) initiated the reaction, incubated at 25 ± 2°C for 15 min, then absorbance was measured at λ_{max} 560 nm. The scavenging activity of O_2^- was calculated by Equation 1.

Inhibition of lipid peroxidation in rat liver homogenate

Inhibition of lipid peroxidation activity of extracts was determined by measuring the intensity of pink colored complex formed by the reaction between malondialdehyde and TBA.[32,33] Swiss albino rats (180-240 g) of either sex were sacrificed, and their livers were dissected out, washed properly with potassium chloride solution (0.15 M), homogenized and centrifuged at 3000 rpm for 10 min at 40 ± 2°C and discarded the precipitate. In liver homogenate (1 mL, 1% w/v), ferrous chloride ($FeCl_2$) (0.5 mL, 0.5 mM), H_2O_2 (0.5 mL, 0.5 mM), and various concentrations of extracts or vitamin C (1 mL, 20-150 µg/mL) were added and incubated at 37 ± 2°C for 60 min. After incubation 1 mL of each trichloroacetic acid (15%) and TBA (0.67%) were added in reaction mixture, heated on boiling water bath for 25 min. Intensity of pink color formed was measured at λ_{max} 535 nm. Percent inhibition of LPO was calculated using Equation 1.

Determination of Hepatoprotective Effect

In vitro hepatoprotective effect on HepG2 cell line against CCl_4-induced damage

Human live hepatoma cells (HepG2) were procured from National Center for Cell Science, Pune, India. The HepG2 cells (1×10^5 cells/T25 flask) were seeded and cultured in DMEM containing fetal bovine serum (10%), penicillin (100 IU/mL) and streptomycin (0.1 µg/mL) and incubated in an humidified 5% CO_2 atmosphere at 37 ± 2°C for 24 h for more than 80% confluence. Cells were passaged by trypsinization of sub-confluent culture using trypsin phosphate versene glucose solution containing trypsin (0.2% w/v), EDTA (0.02% w/v), and glucose (0.05% w/v) in phosphate buffer saline.

The cell viability determined by 3-(4,5 dimethyl thiazole-2 yl)-2,5-diphenyl tetrazolium bromide (methylthiazole tetrazolium [MTT]) cytotoxicity assay is based on cleavage of tetrazolium salt to a blue formazan derivative by living cells and reflects the mitochondrial activity.[34] Briefly, HepG2 cells (3×10^6 cells/well) were maintained in different groups in 96 well culture plates. Solutions of CCl_4 (1% v/v), silymarin, and extracts were prepared in serum free DMEM containing DMSO (0.1% v/v). After removing the media normal control group (Group I) cells were treated with 100 µl serum free DMEM containing DMSO (0.1% v/v) while toxicant control (Group II) and standard control (Group III) were treated with 100 µl of CCl_4 (1% v/v) and 100 µl silymarin of varying concentrations (50,100, 150, 200, 250 µg/mL), respectively. Treatment groups (Group IV-VIII) cells were treated with 100 µl of $CCl_{4(}$1% v/v) and 100 µl of PEE, CHE, EAE, EtOHE and AQE with different concentrations (50,100, 150, 200, 250 µg/mL), respectively. After 2 h, serum free DMEM (100 µl) was added in each group for next 24 h. At the end of incubation period, MTT solution (20 µl, 5 mg/mL) was added, shake gently and incubated for 3 h in 5% CO_2 atmosphere at 37 ± 2°C and supernatant was removed. MTT is reduced by mitochondrial dehydrogenase activity in metabolically active cells to form insoluble formazan crystals. Then, isopropanol (50 µL) was added for solubilizing the formazan crystals. Absorbance was measured at 540 nm using microplate reader (Thermo Scientific, USA). The absorbance given by untreated cells was taken as 100% cell survival, and the relative (%) cell viability was calculated using following formula:

$$\% \text{ Cell viability} = (Abs_{control} - Abs_{sample})/Abs_{control} \times 100$$

Where, $Abs_{control}$ is the absorbance of control sample and Abs_{sample} is the absorbance of test sample.

In vivo hepatoprotective effects

Animals

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

Acute oral toxicity

Acute oral toxicity was performed according to Organization for Economic Co-operation and Development guideline

No. 423.[35] Female rat fasted overnight, accessing water *ad libitum* were used in this study. The extract was administered orally at a dose of 300 mg/kg body weight, and the animals were observed for mortality or any abnormal behavior for first 24 h, then for up to next 14 days. The procedure was repeated at a dose of 2000 mg/kg body weight to determine the median lethal dose.

Direct method

Swiss albino rats used for this study were divided into 18 groups containing six rats in each group. The CCl_4 is diluted in olive oil (1:1) while suspension of silymarin as well as extracts were prepared in 2% v/v solution of Tween 80, which were used in prophylactic and curative studies.[36,37]

Prophylactic study

Group I received only the vehicle, Tween 80 (1 mL/kg body weight/day) orally for 12 days. Groups II-XVIII received orally Tween 80 (1 mL/kg body weight/day), silymarin (100 mg/kg body weight/day) as well as the extracts at dose of 100, 200 and 400 mg/kg body weight, respectively for the first 7 days followed by intraperitoneal (i.p.) injections of CCl_4 in olive oil (1:1) at a dose of 0.5 mL/kg body weight for the next 5 days (i.e. a total of 12 days). The treatments (vehicle, silymarin, and extracts) were given daily while CCl_4 was given on alternate days, i.e. on 8th, 10th and 12th day.

Curative study

Group I received only the vehicle, Tween 80 (1 mL/kg body weight/day) orally for 12 days. Groups II-XVIII received i.p. injection of CCl_4 in olive oil (1:1) at a dose of 0.5 mL/kg body weight for 5 days and then orally administered Tween 80 (1 mL/kg body weight/day), silymarin (100 mg/kg body weight/day), as well as the extracts at dose of 100, 200 and 400 mg/kg body weight, respectively, for next 7 days (i.e. a total of 12 days). The treatments (vehicle, silymarin, and extracts) were given daily while CCl_4 was given on alternate days, i.e. on 1st, 3rd and 5th day.

Hepatoprotective effect (H) expressed as percent hepatoprotection was calculated using following equation:

$$H = 1 - (T-V/C-V) \times 100$$

Where, T is the mean value of drug and CCl_4 treatment, C is the mean value of CCl_4 treatment alone and V is the mean value of vehicle treatment.

Assessment of hepatoprotective activity

The animals receiving different treatments were euthanized on the 13th day and blood samples were collected by retro-orbital puncturing method. The serum was separated by centrifugation at 1000 rpm for 10 min and analyzed for various biochemical parameters including serum glutamate oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT), alkaline phosphatase (ALP), total bilirubin (TB), triglycerides (TGs), and cholesterol using Erba Chem 5 Plus V2 biochemistry analyzer (Transasia, Germany).

Histopathological examination

Livers of rats receiving different treatments were isolated, fixed in formaldehyde (10% v/v) and histopathologically examined using hematoxylin and eosin dyes. The cellular images were captured at suitable magnification.

Indirect method (Barbiturate-induced sleep model)

This method was used for both curative and prophylactic studies. On the 13th day, all the animals received pentobarbitone sodium (25 mg/kg body weight, i.p.) and were observed for sleep. The time interval between loss and regain of righting reflex was recorded as PST for each animal.[37,38]

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test using Graph Pad Instat Software (Version 5.0, Graph Pad Software, San Diego, California, USA). A probability $P \leq 0.05$ was considered as significant. The results were expressed as mean ± standard error of mean ($n = 3$) or specified in the study.

Estimation of quercetin in Z. xylopyrus leaves extracts using reversed-phase high-performance liquid chromatography (RP-HPLC)

Amount of quercetin in different extracts of *Z. xylopyrus* leaves was estimated by isocratic RP-HPLC (Shimadzu, Japan) comprising UV-Vis detector, RP-C18 column (250 mm × 4.6 mm, 5 µm) and spinchrome software.[39]

Mixture of methanol/phosphoric acid (0.2%) (65:35 v/v) was used as the mobile phase at a flow rate of 1 mL/min at room temperature. Quercetin was quantified by UV-Vis detector following RP-HPLC separation at 360 nm.

RESULTS

Determination of Phytoconstituents

Preliminary phytochemical screening

Results of preliminary phytochemical screening were showed in Table 1.

Estimation of TPC, TFC, and TTC

Results demonstrated that amount of TPC, TFC, and TTC differed significantly among various extracts as given in Table 2. The TPC and TFC were found more in EAE 194.16 ± 0.74 mg GAE/g, 43.7 ± 0.78 mg QE/g of extract, respectively, while TTC was found significantly more in EtOHE 54.84 ± 1.42 mg GAE/g of extract among all the extracts. However, this method has some limitation as FCR reacts with other non-phenolic reducing compounds like organic acids, sugar leading to over evaluation of phenolic compound.[40]

Estimation of Antioxidant Activity

FRAP assay

The ferric ion reducing power of extracts was represented in Figure 1a. The reducing ability of different extracts showed following order - Vitamin C > EAE > EtOHE > PEE > CHE > AQE.

DPPH radical scavenging assay

DPPH radical scavenging assay measured hydrogen donating nature of extracts.[41] Under DPPH radical scavenging activity the inhibitory concentration 50% (IC_{50}) value of PEE, CHE, EAE, EtOHE and AQE extract was found to be 347.44 ± 2.78, 298.25 ± 1.86, 103.50 ± 2.05, 125.19 ± 1.83 and 257.02 ± 3.52 µg/mL, respectively, as compared to that of vitamin C (11.29 ± 0.66 µg/mL) [Table 3 and Figure 1b].

Hydroxyl radical scavenging assay

$OH^{\cdot}$ radical is one of the most reactive radicals, which has the capacity to conjugate with the nucleotides of DNA resulting in the strand breakage and leads to carcinogenesis, mutagenesis, and cytotoxicity.[26] However, the presence of antioxidant prevents the cellular damage either by quenching off or chelating transition metals.[42] The PEE, CHE, EAE, EtOHE and AQE extracts showed $OH^{\cdot}$ radical scavenging activity with IC_{50} value of 346.17 ± 3.24, 241.55 ± 1.43, 89.33 ± 1.79, 122.95 ± 2.89 and 306.24 ± 2.65 µg/mL. Vitamin C showed an excellent (IC_{50} 17.99 ± 1.42 µg/mL) activity. EAE showed significant activity as compared to other extracts [Table 3 and Figure 1c].

Table 1: Phytochemical screening of *Z. xylopyrus* leaves extract

Phytoconstituents	PEE	CHE	EAE	EtOHE	AQE
Alkaloids	-	-	-	+	-
Glycosides	-	-	+	+	-
Flavonoids	-	-	+	+	-
Tannins	-	-	+	+	-
Fixed oils/fat	-	-	-	-	-
Carbohydrate	-	-	-	+	+
Saponins	-	-	-	-	+
Steroids	-	+	+	-	-
Terpenoids	+	+	-	-	-
Amino acids	-	-	-	-	-
Gum and mucilage	-	-	-	-	-

Where (+) present, (-) absent. PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract

Nitric oxide ($NO^{\cdot}$) radical scavenging assay

Extracts showed $NO^{\cdot}$ scavenging effects by competing with oxygen to react with NO directly hence inhibited the nitrite ion formation.[28] PEE, CHE, EAE, EtOHE and AQE extracts showed nitric oxide ($NO^{\cdot}$) radical scavenging activity with IC_{50} value of 425.55 ± 4.59, 402.62 ± 2.36, 129.34 ± 1.29, 144.67 ± 1.86 and 270.58 ± 2.87 µg/mL, respectively, as compared to that of vitamin C (IC_{50} 26.21 ± 0.86 µg/mL). EAE showed significant activity as compared to other extracts [Table 3 and Figure 1d].

Superoxide radical activity assay

Superoxide anion plays an important role in the formation of other reactive oxygen species such as H_2O_2, $OH^{\cdot}$ and singlet oxygen, which induced oxidative damage in lipid, protein and DNA.[43] The O_2^{-} radical scavenging activity of PEE, CHE, EAE, EtOHE and AQE in terms of IC_{50} value was found to be 365.43 ± 3.67, 347.44 ± 1.22, 62.03 ± 1.29, 110.47 ± 1.48 and 273.56 ± 3.29 µg/mL, respectively, as compared to that of vitamin C (34.35 ± 0.78 µg/mL). EAE exhibited significant activity in comparison to other extracts [Table 3 and Figure 1e].

Inhibition of lipid peroxidation assay

The PEE, CHE, EAE, EtOHE, AQE and vitamin C showed inhibition of lipid peroxidation with IC_{50} value of 372.51 ± 3.84, 344.02 ± 2.89, 110.05 ± 2.96, 136.26 ± 1.76, 279.93 ± 2.81 and 71.15 ± 1.89 µg/mL, respectively [Table 3 and Figure 1f]. EAE extract demonstrated significant inhibition of LPO as compared to other extracts.

Table 2: Estimation of TPC, TFC and TTC of *Z. xylopyrus* leaves extracts

Extracts	TFC (mg QE/g of extract)	TPC (mg GAE/g of extract)	TTC (mg GAE/g of extract)
PEE	3.45±1.14	20.64±0.22	6.54±0.82
CHE	5.24±0.88	33.24±0.16	8.46±1.48
EAE	43.76±0.78	194.16±0.74	20.45±2.31
EtOHE	18.07±2.45	138.42±0.82	54.84±1.42
AQE	4.11±1.11	26.40±0.47	13.16±2.19

Values are represented as mean±SEM, *n*=3. SEM: Standard error of mean, TFC: Total flavonoids content, TPC: Total phenolic content, TTC: Total tannin content, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract

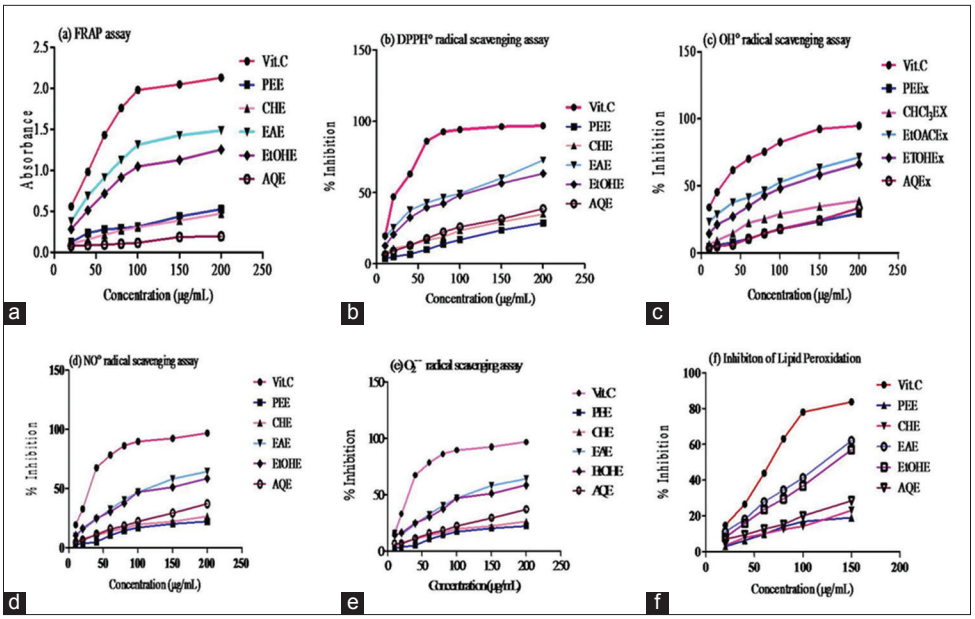

Figure 1: Estimation of antioxidant activity of *Zizyphus xylopyrus* leaves extracts by different assays; (a) Ferric reducing ability of plasma assay, (b) 2,2-diphenyl-1-picrylhydrazyl° radical scavenging assay, (c) OH° radical scavenging assay, (d) NO° radical scavenging assay, (e) O_2^- scavenging assay, and (f) inhibition of lipid peroxidation. Values are represented as mean ± SEM; (n=3)

Table 3: Antioxidant activity of *Z. xylopyrus* leaves extracts in terms of IC_{50} value

Assay	IC_{50} (µg/mL)					
	Extracts					
	Vitamin C	PEE	CHE	EAE	EtOHE	AQE
DPPH°	11.29±0.66	347.44±2.78	298.25±1.86	103.50±2.05	125.19±1.83	257.02±3.52
OH°	17.99±1.42	346.17±3.24	241.55±1.43	89.33±1.79	122.95±2.89	306.24±2.65
NO°	26.21±0.86	425.55±4.59	402.62±2.36	129.34±1.29	144.67±1.86	270.58±2.87
O_2^-	34.35±0.78	365.43±3.67	347.44±1.22	62.03±2.78	110.47±1.48	273.56±3.29
Inhibition of LPO	71.15±1.89	372.51±3.84	344.02±2.89	110.05±2.96	136.26±1.76	279.93±2.81

Values are represented as mean±SEM, *n*=3. SEM: Standard error of mean, DPPH: 2,2-diphenyl-1-picrylhydrazyl, LPO: Lipid peroxidation, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract

Hepatoprotective Effects

In vitro hepatoprotective effect on HepG2 cell line against CCl_4 induced damage

HepG2 cell on treatment with CCl_4 (1% v/v) alone exhibited significant cell death. The cell viability was found to be 25.30 ± 1.34%. While on treatment with EAE extract along with CCl_4, cell viability was significantly ($P < 0.001$) increased. The increased cell viability (maximum 89.97 ± 2.68%) was observed by increasing the concentration, i.e., at the dose of 250 µg/mL of EAE, which is comparable to standard silymarin (95.23 ± 1.66%) treatment with similar dose. The EtOHE treated cells showed only 67.37 ± 2.56% cell viability while treatment with PEE, CHE and AQE did not show the marked protective effect against CCl_4-induced cell death [Table 4].

In vivo hepatoprotective effects

Acute toxicity studies

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

Direct prophylactic and curative effect

Study results showed that treatment with the extract restores/decreased the elevated serum enzyme levels produced by CCl_4-induced hepatic damage, which might be due to cell membrane stabilization, repair of damaged hepatic tissue and/or antioxidant activity of the extract.

In case of both prophylactic and curative treatment, EAE extract more significantly ($P < 0.001$) decreased the elevated

Table 4: *In vitro* hepatoprotective effect on HepG2 cell line against CCl_4-induced damage

Group	Concentration (µg/mL)		% Cell viability	IC_{50} (µg/mL)
Group I (Normal control)	-	-	100.3±0.40***	-
Group II (Toxicant control)	-	CCl_4 (1% v/v)	25.30±1.34	-
Group III (Positive control) (Silymarin treated)	50		31.88±1.97*	76.11±0.96
	100		58.65±2.28***	
	150	CCl_4 (1% v/v)	90.46±2.59***	
	200		93.39±1.37***	
	250		95.23±1.66***	
Group IV (PEE treated)	50		26.30±1.03	1724.28±3.48
	100		27.37±1.18	
	150	CCl_4 (1% v/v)	28.37±1.52	
	200		28.9±1.23	
	250		29.10±0.40	
Group V (CHE treated)	50		26.31±0.83	656.92±2.69
	100		28.79±1.62	
	150	CCl_4 (1% v/v)	29.79±0.67	
	200		31.77±1.58*	
	250		34.60±1.82*	
Group VI (EAE treated)	50		29.3±1.06	80.93±1.02
	100		63.93±0.85***	
	150	CCl_4 (1% v/v)	79.67±2.03***	
	200		87.33±2.31***	
	250		89.97±2.68***	
Group VII (EtOHE treated)	50		27.4±0.43	144.39±1.83
	100		42.17±1.25***	
	150	CCl_4 (1% v/v)	53.20±2.67***	
	200		65.87±3.39***	
	250		67.37±2.56***	
Group VIII (AQE treated)	50		25.63±0.56	1402.77±4.06
	100		26.37±1.52	
	150	CCl_4 (1% v/v)	28.0±1.77	
	200		28.90±1.23	
	250		29.1±0.40	

Values are represented as mean±SEM, n=3, *P<0.05, ***P<0.001 versus toxicant control group (CCl_4). SEM: Standard error of mean, CCl_4: Carbon tetrachloride, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract, IC_{50}: Inhibitory concentration 50%

enzyme levels of SGOT, SGPT, ALP, TB, TG and cholesterol as compared to that of EtOHE and were found comparable to the standard control, silymarin ($P < 0.001$) while PEE, CHE and AQE did not exhibit marked effect on serum enzyme levels [Tables 5 and 6].

The increased hepatic protection was observed by increasing the dose of the extract, which was further supported by histopathological examination [Figures 2 and 3].

Indirect method (pentobarbitone-induced sleeping time [PST])

In case of both, prophylactic and curative effect, the treatment with EAE showed more significant ($P < 0.001$) reduction in PST in rats than that of EtOHE ($P < 0.05$), which was eventually comparable to that observed in silymarin treated group. On the other hand the PEE, CHE and AQE did not show any marked effect on PST [Table 7].

Table 5: Prophylactic effect of *Z. xylopyrus* leaves extracts against CCl4-induced hepatic damage

Groups	Dose (mg/kg, p.o.)	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TB (mg/dL)	TG (mg/dL)	Cholesterol (mg/dL)
Group I (Normal control)	-	109.2±5.86***	71.63±7.91***	135.6±5.30***	1.75±0.14***	98.77±4.51***	75.20±5.98***
Group II (Toxicant control)	CCl_4 (0.5 mL/kg, i.p.)	652.5±14.97	353.8±12.29	342.8±8.08	7.45±0.16	196.6±7.10	147.4±5.98
Group III (Standard control) (Silymarin treated)	100	248.7±10.82*** (74.32)	149.9±9.50*** (72.26)	174.1±9.16*** (81.41)	3.63±0.20*** (67.01)	121.8±6.52*** (76.45)	99.84±5.92*** (65.87)
Group IV-VI (PEE treated)	100	641.8±8.58 (2.11)	348.9±10.36 (1.73)	338.2±8.88 (2.22)	7.31±0.16 (2.45)	193.3±6.08 (3.37)	139.8±6.08 (10.52)
	200	634±13.62 (3.40)	342.8±6.73 (3.89)	333.2±9.13 (4.63)	6.95±0.10 (8.77)	191.4±5.00 (5.31)	134.7±7.99 (17.59)
	400	627.7±11.96 (4.56)	328.2±7.05 (9.07)	305.6±10.53* (17.97)	6.70±0.23 (13.15)	180.3±5.44 (16.66)	130.8±5.42 (22.99)
Group VII-IX (CHE treated)	100	638.7±9.59 (2.54)	347.2±8.41 (2.33)	340.1±9.76 (1.30)	7.18±0.13 (4.73)	194.9±4.68 (1.73)	138.9±5.84 (11.77)
	200	607.4±10.21* (8.30)	343.9±6.8 (3.50)	330.3±8.51 (6.03)	6.85±0.20 (10.52)	187.8±7.15 (8.99)	123.5±8.65 (33.10)
	400	586.9±10.01** (12.04)	316.5±8.39* (13.21)	320.2±9.67 (10.90)	6.66±0.14* (13.85)	182.2±6.35 (14.71)	116.7±5.19 (42.52)
Group X-XII (EAE treated)	100	601.3±10.28** (9.42)	304.6±8.57** (17.43)	298.6±6.25** (21.33)	6.45±0.28* (17.54)	167.8±5.14** (29.43)	126.6±6.38* (28.78)
	200	381.7±9.23*** (49.84)	246.2±7.93*** (38.13)	250.6±4.60*** (44.49)	5.26±0.27*** (38.42)	147.8±4.33*** (49.88)	112.3±5.44** (48.61)
	400	283.6±8.19*** (67.9)	169.8±6.16*** (65.20)	190.7±9.53*** (76.01)	4.28±0.19*** (55.61)	130.6±5.71*** (67.46)	103.7±7.20*** (60.40)
Group XIII-XV (EtOHE treated)	100	602.4±11.2* (9.22)	318.4±9.09 (12.54)	305.4±10.74* (18.05)	6.61±0.17* (14.73)	172.1±6.49* (25.04)	127.7±5.59* (27.28)
	200	475±5.05*** (32.67)	282.4±10.61*** (25.30)	275.5±6.53*** (32.48)	5.95±0.19*** (26.31)	160.5±6.31*** (36.90)	119.1±4.05* (39.19)
	400	397.4±9.99*** (48.05)	230.7±12.90*** (43.62)	240.4±9.74*** (49.42)	4.56±0.26*** (50.77)	152.1±4.11*** (45.48)	109.5±7.06* (52.49)
GroupXVI-XVIII (AQE treated)	100	634.9±11.86 (3.23)	340.2±11.06 (4.81)	332.2±10.95 (5.11)	7.56±0.13 (−1.92)	194.6±8.02 (2.04)	137.2±6.55 (14.12)
	200	637.3±12.26 (2.79)	336.2±9.46 (6.23)	330.8±6.29 (5.79)	6.40±0.16** (18.42)	180.6±4.35 (16.35)	132.1±4.79 (21.06)
	400	631.3±10.72 (3.90)	334.9±8.73 (6.69)	318.3±7.81 (11.82)	6.86±0.22 (10.35)	183.4±5.81* (13.49)	127.6±4.79* (27.42)

Values are represented as mean±SEM, n=6, *P<0.05, **P<0.01, ***P<0.001 versus Toxicant control group (CCl_4). Values in parentheses indicate percent hepatoprotection (H). CCl_4: Carbon tetrachloride, SEM: Standard error of mean, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract, *Z. xylopyrus: Zizyyphus xylopyrusi,* SGOT: Serum glutamate oxaloacetate transaminases, SGPT: Serum glutamate pyruvate transaminases, ALP: Alkaline phosphatase, TB: Total bilirubin, TG: Triglycerides

Estimation of Quercetin in *Z. xylopyrus* Leaves Extracts Using RP-HPLC

Concentration of quercetin in various extracts has been determined by standard curve of quercetin using regression equation y = 119.4x + 45.84, r^2 = 0.998, where y is the area under peak and x is concentration. Maximum amount of the quercetin was found in EAE (32.8 ± 0.24 mg/g of dried extract) followed by EtOHE (7.2 ± 0.09 mg/g of dried extract). PEE does not contain quercetin while CHE and AQE showed the presence of small amount of quercetin [Table 8 and Figure 4].

Table 6: Curative effect of *Z. xylopyrus* leaves extracts against CCl_4-induced hepatic damage

Groups	Dose (mg/kg, p.o.)	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TB (mg/dL)	TG (mg/dL)	Cholesterol (mg/dL)
Group I (Normal control)	-	109.21±5.86***	71.63±7.91***	135.6±5.30***	1.75±0.14***	98.77±4.51***	75.2±5.98***
Group II (Toxicant control)	CCl_4 (0.5 mL/kg, i.p.)	446.9±11.34	151.3±7.09	248.6±10.71	4.8±0.28	171.2±7.49	128.1±7.58
Group III (Standard control) (Silymarin treated)	100	237.6±9.03*** (61.97)	104.8±6.56*** (58.36)	162.7±10.82*** (76.01)	2.4±0.18*** (78.68)	102.38±7.98** (81.2)	92.05±7.63*** (68.15)
Group IV-VI (PEE treated)	100	442.1±10.66 (1.42)	144.7±8.19 (8.28)	248.2±10.58 (0.35)	4.66±0.22 (4.59)	163.5±5.98 (10.63)	120.9±5.53 (13.61)
	200	428.90±11.45 (5.33)	138.6±7.25 (15.94)	234.3±7.31 (12.65)	4.43±0.18 (12.13)	162±8.87 (12.6)	116.6±4.64 (21.73)
	400	419.6±10.64 (8.08)	129.9±6.30 (26.86)	229.5±8.16 (16.90)	4.38±0.20 (13.77)	157.65±7.85 (18.7)	115.4±6.7 (24.0)
Group VII-IX (CHE treated)	100	422.2±10.82 (7.31)	151.7±6.72 (–0.50)	235.8±7.13 (11.33)	4.7±0.24 (3.27)	165.6±9.14 (7.6)	118.9±6.87 (17.39)
	200	410.7±8.36 (10.71)	143.4±5.77 (9.91)	228.7±6.50 (17.61)	4.36±0.15 (14.42)	162.9±7.10 (11.4)	109.5±7.93 (35.16)
	400	399.0±7.15* (14.18)	124.1±5.58* (34.14)	217.7±6.20 (27.34)	4.1±0.12 (22.95)	156.8±5.17* (19.8)	100.7±5.89* (51.79)
Group X-XII (EAE treated)	100	404.8±9.13* (12.46)	124.2±5.10* (34.01)	211.2±6.50* (33.09)	3.86±0.08* (30.82)	139.6±9.58 (35.16)	109.5±9.58* (35.16)
	200	309.4±11.59*** (40.71)	115.1±5.89** (45.43)	200.9±7.60** (42.21)	2.78±0.21*** (66.23)	130.6±5.93* (56.05)	100.9±5.69** (51.52)
	400	260.1±9.33*** (55.31)	109.9±8.36*** (51.96)	173.5±6.84*** (66.46)	2.53±0.16*** (74.43)	118.76±6.55*** (72.4)	93.18±4.12*** (66.13)
Group XIII-XV (EtOHE treated)	100	397.4±12.08** (14.65)	136.4±6.04 (18.70)	224.1±12.02 (21.68)	4.11±0.17 (22.62)	152.3±9.20 (26.09)	116.3±5.26 (22.30)
	200	334.0±12.08*** (33.43)	121.5±6.81* (37.40)	207.2±7.23** (36.63)	3.9±0.22* (29.52)	141.0±6.52** (41.69)	101.8±4.96* (49.71)
	400	288.2±11.3*** (46.99)	117.6±9.22* (42.29)	198.6±6.20*** (44.24)	3.76±0.19** (34.10)	136.3±7.80* (48.18)	96.58±7.57*** (59.58)
GroupXVI-XVIII (AQE treated)	100	434.81±10.21 (3.58)	144.1±5.14 (9.03)	237.8±12.36 (9.55)	4.36±0.16 (14.43)	166.4±7.95 (6.62)	119.9±5.84 (15.50)
	200	420.1±8.87 (7.93)	143.9±7.61 (9.28)	225.5±9.67 (20.44)	4.2±0.20 (19.67)	159.5±5.52 (16.02)	115.5±3.97 (12.6)
	400	411.7±8.53 (10.42)	137.8±5.18 (16.94)	215.8±8.24 (29.02)	3.93±0.18* (28.52)	153.8±4.96 (24.02)	118.4±7.50** (18.33)

Values are represented as mean±SEM, n=6, *P<0.05, **P<0.01, ***P<0.001 versus toxicant control group (CCl_4). Values in parentheses indicate percent hepatoprotective activity (H). SEM: Standard error of mean, CCl_4: Carbon tetrachloride, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract, *Z. xylopyrus: Zizyphus xylopyrus*, SGOT: Serum glutamate oxaloacetate transaminases, SGPT: Serum glutamate pyruvate transaminases, ALP: Alkaline phosphatase, TB: Total bilirubin, TG: Triglycerides

DISCUSSION

Oxidative stress is a result of physiological imbalance between pro-oxidant and antioxidant disruption.[1] However, biological system of our body protects us against free radical and ROS-induced damage via endogenous production of antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase and antioxidant nutrients such as ascorbic acid, α-tocopherol and glutathione.[27] However, the overproduction of ROS or imbalance between system's ability to detoxify toxic intermediate or repair the damage plays a vital role in LPO, which leads to hepatic

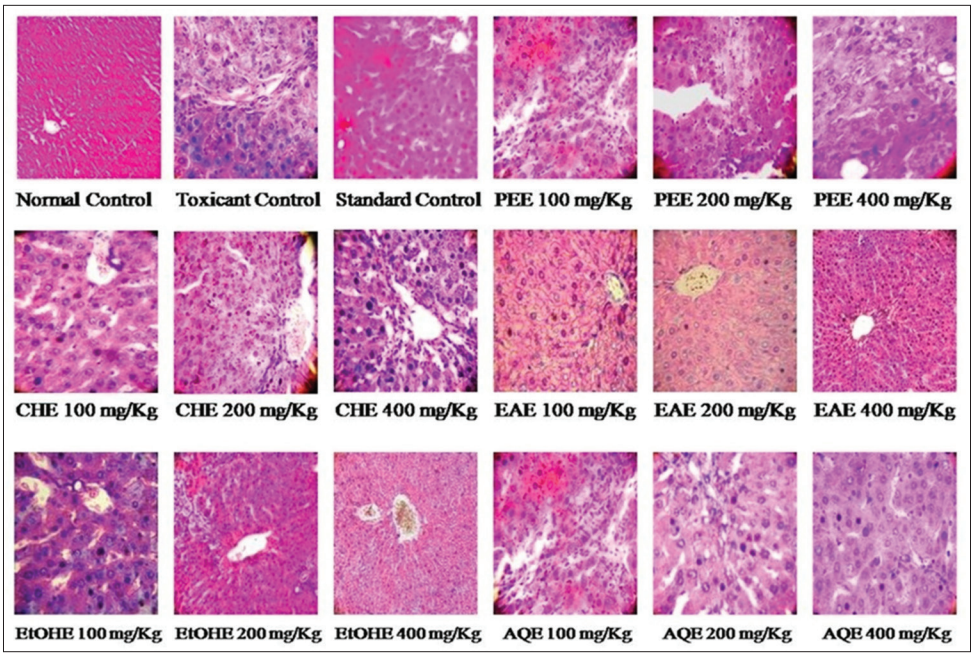

Figure 2: Prophylactic effects of *Zizyphus xylopyrus* leaves extracts against carbon tetrachloride-induced hepatic damage

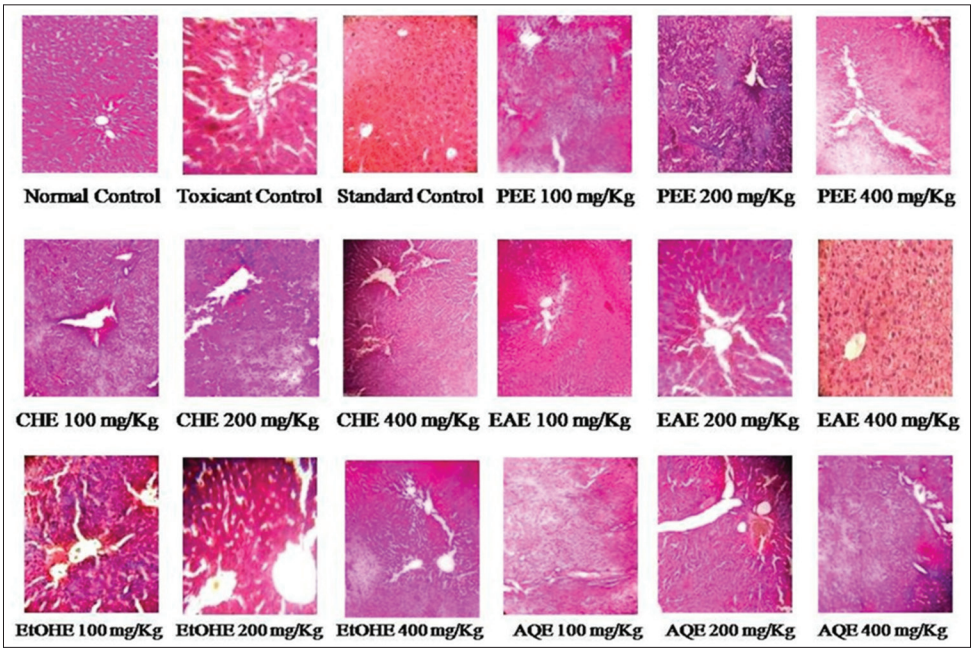

Figure 3: Curative effects of *Zizyphus xylopyrus* against carbon tetrachloride-induced hepatic damage

dysfunction.[42] Therefore the present study was designed to investigate the hepatoprotective potential of different extract of *Z. xylopyrus* leaves against CCl_4-induced toxicity by *in vitro* and *in vivo* models. Polyphenols are the major category of phytoconstituents with antioxidant activity, which is mainly attributed due to their redox properties, i.e. absorbing and neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides.[28,44]

Preliminary phytochemical screening results showed the presence of alkaloids, flavonoids, tannins, terpenoids, steroids and carbohydrate in various extracts of *Z. xylopyrus* while EAE showed the presence of flavonoids, tannins, steroids, and terpenoids. These compounds have been previously reported to possess antioxidant as well as hepatoprotective potential.[44] In this study, antioxidant activity of different *Z. xylopyrus* leaves extracts have been determined by different *in vitro* methods like FRAP assay, DPPH$^{\bullet}$, OH$^{\bullet}$, NO$^{\bullet}$, and O_2^- radical scavenging assay. *In vitro* antioxidant activity results demonstrated that among all extracts, EAE demonstrated significantly less IC_{50} value or more antioxidant activity among all extracts, which was comparable to vitamin C. Reducing property of extracts is generally associated with the presence of reductants, which break off free radical

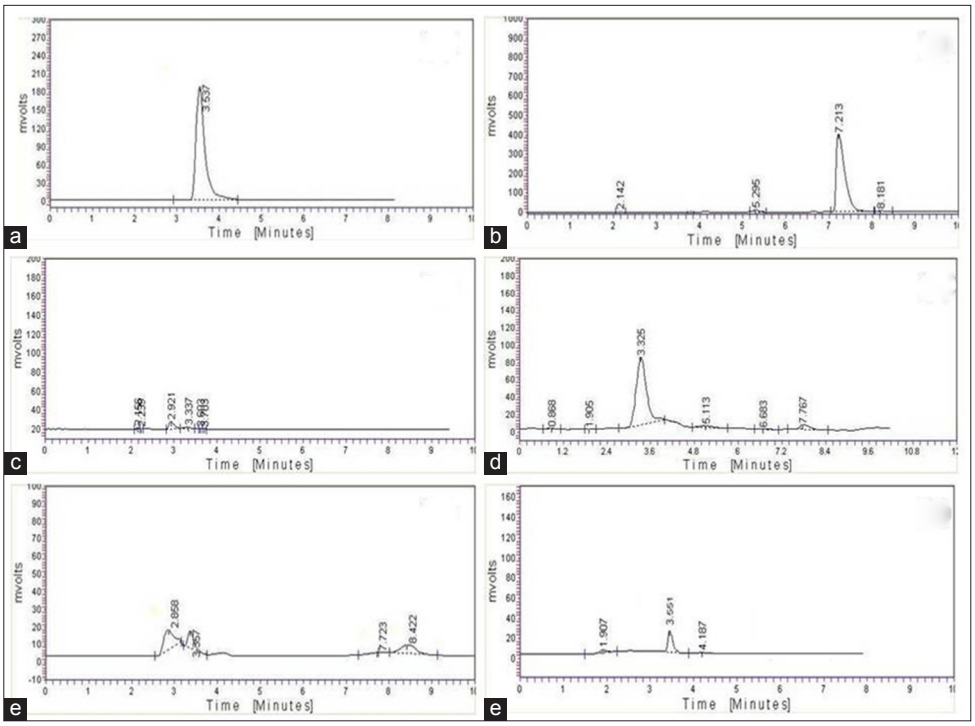

Figure 4: Estimation of quercetin in *Zizyphus xylopyrus* leaves extracts by reversed-phase high-performance liquid chromatography (HPLC) (a) HPLC chromatogram of standard quercetin, (b) HPLC chromatogram of petroleum ether extract, (c) HPLC chromatogram of chloroform extract, (d) HPLC chromatogram of ethyl acetate extract, (e) HPLC chromatogram of ethanol extract, (f) HPLC chromatogram of aqueous extract

Table 7: Effect of *Z. xylopyrus* leaves extracts on pentobarbitone induced sleep

Group	Dose (mg/kg, p.o.)	Prophylactic effects		Curative effects	
		Time of recovery (Min.)	% Recovery	Time of recovery (Min.)	% Recovery
Group I (Normal control)	-	51.17±4.131***	-	51.17±4.13***	
Group II (Toxicant control)	CCl_4 (0.5 mL/kg, i.p.)	117.9±8.17	-	142.9±6.94	
Group III (Standard control) (Silymarin treated)	100	86.24±3.63**	40.05	106.1±5.64***	47.44
Group IV-VI (PEE treated)	100	114.6±8.80	4.94	136.1±4.67	7.41
	200	110.9±5.89	10.49	127.8±5.98	16.46
	400	105.4±4.29	18.73	138±7.63	5.34
Group VII-IX (CHE treated)	100	112.7±3.83	7.79	133.9±7.14	9.81
	200	116.9±4.38	1.49	130±5.95	14.06
	400	105±4.81	19.33	123.5±7.33	21.14
Group X-XII (EAE treated)	100	111.4±6.35	9.74	126.1±4.56	18.31
	200	87.1±7.93**	46.08	112.2±3.57**	33.46
	400	78.9±7.25***	58.31	99.25±5.76***	47.58
Group XIII-XV (EtOHE treated)	100	108.8±5.27	13.64	132.4±8.35	11.44
	200	91.95±5.04*	38.88	115.2±5.52*	30.19
	400	88.24±6.73*	44.45	109.4±6.22**	36.52
Group XVI-XVIII (AQE treated)	100	115.4±9.07	3.75	139.8±5.19	3.37
	200	114.2±7.93	5.54	135.3±4.51	8.28
	400	100.2±3.39	26.52	132.9±8.05	10.9

Values are represented as mean±SEM, n=6, *P<0.05, **P<0.01, ***P<0.001 versus toxicant control group (CCl_4). PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract, SEM: Standard error of mean, CCl_4: Carbon tetrachloride

Table 8: Estimation of quercetin in *Z. xylopyrus* leaves extracts using RP-HPLC

Standard quercetin		Regression equation	Extracts	Concentration of quercetin (mg/g)
Concentration (µg/mL)	Area			
5	609.49±0.75	y=119.4x+45.84 r^2=0.998	PEE	-
10	1251.77±0.94		CHE	0.8±0.06
15	1863.58±1.32		EAE	32.8±0.24
20	2483.28±1.72		EtOHE	7.2±0.09
25	2979.84±0.31		AQE	1.9±0.17

*Data are expressed as means±SD, *n*=3. SD: Standard deviation, RP-HPLC: Reversed-phase high-performance liquid chromatography, PEE: Petroleum ether extract, CHE: Chloroform extract, EAE: Ethyl acetate extract, EtOHE: Ethanol extract AQE: Aqueous extract

chain reaction by donating hydrogen atom. The marked antioxidant activity of EAE was probably due to the presence of phenolic components, which acted as reductants, i.e. due to its hydrogen donating nature.[26]

The EAE significantly reduced LPO induced by $FeCl_2$-H_2O_2 (IC_{50} 110.05 ± 2.96 µg/mL). The LPO inhibition capacity of the extract might be due to OH• radical scavenging activity of the extract, which might be due to presence of polyphenols (TPC 194.16 ± 0.74 mg GAE/g of dried extract and TFC 43.76 ± 0.78 mg QE/g of dried extract), possessing phenolic hydroxyl groups, have the ability to accept electrons or combines with free radicals competitively to decrease lipid peroxidation induced by free radicals.[28,33]

Normal hepatocytes are used as *in vitro* model for studying the *in vitro* xenobiotics metabolism and toxicity to the liver.[4] In this study, HepG2 cells were used that minimized any species related differences and maintained majority of specialized function like cell viability, which directly referred the extracellular stress response. Among all the extracts, EAE significantly inhibited the ROS in a dose-dependent manner (IC_{50} 80.93 ± 1.02 µg/mL), which was comparable to silymarin (IC_{50} 76.11 ± 0.96 µg/mL) and resulted in increased cell viability.

Carbon tetrachloride, widely used xenobiotics is metabolized by the liver and produces free radicals (CCl_3• and $OOCCl_3$•), which interfere with the transportation function of the hepatic cells and also react with cellular lipid as well as proteins in the presence of oxygen that leads to leakage of SGOT, SGPT from the cell cytoplasm in serum. The CCl_4 also impairs the excretion of bile from pancreas and consequently increases serum ALP and TB level and altered the cholesterol metabolism and TGs transportation causing increased TG and cholesterol levels leading to fatty liver.[45-47] CCl_4 increased the level of Ca^{2+} in cells, which activated many catabolic enzymes, which destroyed the cytoskeletal construction and cell death through apoptosis or necrosis.[48] In case of both, direct prophylactic and curative study, treatments with EAE (400 mg/kg body weight) significantly preserved the levels of SGOT, SGPT, ALP, TB, TG and cholesterol depicting a marked protective effect in a dose-dependent manner comparable to silymarin treatment.

Damage inflicted by CCl_4 on hepatocytes also caused the loss of microsomal drug metabolizing enzyme of the liver, which resulted in the prolongation of PST. In indirect prophylactic and curative study, treatment with EAE (400 mg/kg) showed a significant reduction in PST, which might be due to improved metabolic capacity of hepatic cells.[36]

The histopathological images depicted that CCl_4-induced the hepatic damage with complete loss of normal cellular structures. The cell hypertrophy, damage in central lobular vein, blood infiltration, fatty changes even necrosis was observed. Histopathological studies provided conclusive evidence that EAE possessed the ability to counteract the CCl_4-induced cytotoxic effects, and advocated the hepatoprotective potential of *Z. xylopyrus* leaves.

On RP-HPLC analysis, the highest content of quercetin (32.8 ± 0.24 mg/g of dry weight of extract) was found to be in EAE followed by EtOHE (7.2 ± 0.09 mg/g in dry weight).

Quercetin is a leading compound for developing the new and effective functional food or medicine due to its remarkable scope of health benefits like antioxidant activities, anti-inflammatory, anti-allergic, anti-cancer, cardioprotective, and hepatoprotective activities.[49] Quercetin showed strong antioxidant activity not only from accepting oxygen free radicals but also by forming metal chelation compounds.[50] The high amount of quercetin present in EAE attenuated the inflammation by down-regulating the CCl_4-induced activation of nuclear factor-kappa B, tumor necrosis factor-α and cyclooxygenase and showed hepatoprotective effects.[51]

CONCLUSION

The results of *in vitro* and *in vivo* studies clearly demonstrated the effectiveness of EAE in attenuation of CCl_4-induced liver damage, possibly by reducing the oxidative stress and inflammation reflecting its antioxidant activity due

to the presence of flavonoids mainly quercetin and other polyphenolic compounds. This study provided experimental evidence, which clearly justified the traditional claims and uses of *Z. xylopyrus* against oxidative liver disease. Since the plant contained quercetin as main active compound along with other polyphenols including flavonoids responsible for hepatoprotective activity as shown by marked antioxidant potential as evidenced by different free radical scavenging assays. Further, the plant extracts were found safe up to 2000 mg/kg body weight in treated rats as witnessed by *in vivo* studies. Hence, we recommend that the *Z. xylopyrus* can be considered for the development of functional food or herbal hepatoprotective medicine after carrying out pre-clinical evaluation and long-term toxicity studies.

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