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Hepatoprotective effect of *Ziziphus oxyphylla* Edgew in paracetamol-induced hepatotoxic rat model

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Abstract: *Ziziphus oxyphylla* Edgew (*Z. oxyphylla*) is a small shrub to a medium sized tree. The aim of the present study was to evaluate the hepatoprotective activity of *Z. oxyphylla* in paracetamol-induced hepatotoxic rat model. Silymarin was used as a standard drug. Methanol extract was found to be the most potent in *in-vitro* antioxidant studies. The methanol extract of *Z. oxyphylla* was administered to experimental animals. The hepatoprotective effect of extract was evaluated by measuring liver biochemical markers, anti-oxidant enzymes and blood lipid profile. Treatment with the paracetamol increased the level of hepatic biomarkers, blood lipid profile and decreased anti-oxidant enzymes. Pre-treatment with *Z. oxyphylla* caused restoration of hepatic biomarkers, blood lipid profile and antioxidant enzymes levels. It is concluded that the methanol extract of *Z. oxyphylla* possesses hepatoprotective activity that might be due to quercetin and kaempferol glycosides present in the plant extract. Further studies are required to elucidate the exact mechanism of action of these isolated flavonoid glycosides.

Keywords: Antioxidant, hepatoprotective, paracetamol, *Ziziphus oxyphylla*.

INTRODUCTION

Plants have been considered as a cornerstone of the traditional medicine systems like Ayurvedic, Unani and Chinese systems (Cragg and Newman, 2013). With the passage of time, interest in plants as a source of medicinal agents has increased. It has been estimated that natural products and their derivatives contributed 50% of the all the drugs used clinically in world. There is still need to study plants being used traditionally in folk medicine for their pharmacological potential and undiscovered chemical entities (Mustafa *et al*., 2017).

Ziziphus oxyphylla Edgew*,* locally known as 'Mamyanu' belongs to a family Rhamnaceae (AJAIB *et al*., 2015). It is a large shrub to a medium sized tree (Abbasi *et al*., 2011). Leaves of the plant have cordate base and their length varies between 2.5 to 6 cm. Ovoid shaped fleshy immature fruits are green while colour changes to red or orange black upon ripening (Ahmad *et al*., 2017). The plant is widely distributed in the warm temperate and subtropical regions throughout the world. It is found in the rainy and mountain areas as well as the Himalayan series of mountains in Pakistan (Khan *et al*., 2015).

Ziziphus oxyphylla has been ethno-medically used to treat hypertension, jaundice, diabetes, pain, allergy, fever, rheumatism, urinary disorders, skin infections, pimples, intestinal worms, digestive disorders, obesity, aches, bleeding gums and hair dandruff (Khan *et al*., 2015; Kaleem *et al*., 2014; AJAIB *et al*., 2015; Abbasi *et al*., 2013). Phytochemical analysis showed the presence of cyclopeptide alkaloids, flavonoids, phenols, phenothiazines, aromatic compounds, amino acids and sulfur compounds (Ahmad *et al*., 2014).

The hepatoprotective effect of the plant has not been studied yet. The aim of the present study was to evaluate the hepatoprotective effect of *Z. oxyphylla* in paracetamol-induced hepatotoxicity animal model. Anti-oxidant tests such as DPPH scavenging assay and hydrogen peroxide of the prepared extracts were performed to estimate the antioxidant activity and the most active extract was selected for *in vivo* studies. Histopatholgical study has been performed to confirm the results of the study.

MATERIALS AND METHODS

Collection of Plant

Ziziphus oxyphylla Edgew was collected from district Bonair of Khyber Pakhtunkhwa, Pakistan in mid-August. Specimen was confirmed by Prof. Dr. Zaheer-ul-Deen at the Department of Botany, Govt. College University, Lahore (Bot. Herb # 3395).

Preparation of plant extracts

The shoots and leaves of the plant were shade dried for 45 days after washing with tap water. *Z. oxyphylla* dried

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parts were ground into the coarse powder for extraction and kept in a well-closed glass bottle. The dried coarse powder of *Z. oxyphylla* plant was extracted by maceration using petroleum ether, chloroform and methanol (Mushtaq *et al*., 2016). The percentage yield was 3.01, 7.45 and 8.31 (w/w%) for petroleum ether, chloroform and methanol extracts respectively.

Antioxidant activities of the prepared extracts

DPPH scavenging assay

One mL of DPPH (0.5 mM) was added in test tubes containing 1 mL of three extracts each and standard separately. After that, 2 ml of the methanol was added in each test tube. By adding 1 mL of DPPH and 3 mL of methanol control sample was prepared. All the test tubes were incubated at room temperature for 30 min and the absorbance was measured at 515 nm. The antioxidant activity (AA) of the three extracts was measured using following formula (Kamali *et al*., 2016):

$$\%AA = \frac{\text{Absorption (Control)} - \text{Absorption (Sample)}}{\text{Absorption (Control)}} \times 100$$

Hydrogen peroxide scavenging assay

Five mL of the sample solution was mixed with 0.6 mL of the hydrogen peroxide solution and absorbance was calculated at 230 nm against a solution of blank. Activity percentage was calculated by using the following formula:

$$\%Activity = \frac{\text{Absorption (Control)} - \text{Absorption (Sample)}}{\text{Absorption (Control)}} \times 100$$

Animals used

Male Wistar albino rats having weight 150-200 g were used. All rats were maintained at twelve hour light/dark cycle and were given pellet diet with water *ad libitum*. In the new environment of the laboratory, the animals were acclimatized for at least 6 to 7 days before starting experiment. The study protocols were approved by University of Sargodha Animal Ethics committee (UOS/ORIC/1441).

Experimental design for paracetamol-induced hepatotoxicity

Rats randomly divided into six groups and each group consisting of six animals, were kept in separate plastic cages. Drugs/extracts were administered orally. Group 1 (Control) received single dose of distilled water for nine days. Group II (Positive control); received distilled water in single dose for nine days and also one dose of paracetamol on eight day (2.5 g/Kg). Group III (Standard drug control); received standard drug (silymarin 100 mg/Kg) for all nine days and on day 8 paracetamol (2.5 g/Kg) two hours after silymarin was administered. Group IV (ZO-100); received methanol extract of *Z. oxyphylla* (100 mg/Kg) for nine days and a single dose of paracetamol (2.5 g/Kg) on day eight, 2 hours after administration of *Z. oxyphylla* extract. Group V (ZO-250); received methanol extract of *Z. oxyphylla* (250 mg/Kg) for nine days and a single dose of paracetamol (2.5 g/Kg) on day eight, 2 hours after administration of *Z. oxyphylla* extract. Group VI (ZO-500); received methanol extract of *Z. oxyphylla* (500 mg/Kg) for nine days and a single dose of paracetamol (2.5 g/Kg) on day eight, 2 hours after administration of *Z. oxyphylla* extract (Mallhi *et al*., 2014).

Blood sample collection and analysis

The animals were anesthetized by chloroform and with the help of needle of disposable syringe, cardiac puncture was made to withdraw the blood (5 ml) on 10^{th} day of the experiment in paracetamol-induced toxicity model. At 4 ºC blood was allowed to clot for twelve hours, and then it was centrifuged at 3000 rpm for 15 min. The collected serum was used for determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total proteins, albumin, bilirubin, triglycerides (TG), cholesterol, low-density lipoproteins (LDL), high density lipoproteins(HDL), glutathione reductase (GR), superoxide dismutase (SOD), malondialdehyde (MDA) and catalase. SOD, GR, MDA and catalase (CAT) were estimated by solid phase enzyme linked immunosorbent assay (ELISA) using automated EIA analyzer (Bio-Rad Laboratories, Hercules, CA, USA) (Raish *et al*., 2016).

Histopathological study

Livers from animals were preserved in 10% neutral buffered formalin. Slides were made by cutting tissues in sections of 4-5 µm thickness and were stained with dye hematoxylin and eosin. These slides were examined under light microscope.

STATISTICAL ANALYSIS

The data were analyzed by one way ANOVA followed by Bonferroni test whereas $P<0.05$ was considered as statistically significant. The GraphPad Prism (version 8.0.2) software has been used for data analysis.

RESULTS

Free radical scavenging ability of different extracts of Z. oxyphylla

Free radical scavenging ability of petroleum ether, chloroform and methanol extracts of *Z. oxyphylla* were assessed by DPPH and hydrogen peroxide scavenging assays. Methanol extract was found to be more effective in all three assays as shown in table 1.

Table 1: Free radical scavenging ability of different extracts of *Ziziphus oxyphylla*

Ziziphus oxyphylla	DPPH assay (% inhibition)	Hydrogen peroxide scavenging assay(% inhibition)
Petroleum ether extract	78.92± 0.23	73.38± 0.63
Chloroform extract	83.19± 0.43	74.98± 1.23
Methanol extract	88.32± 1.03	79.49± 1.43
Vitamin C	99.00± 1.23	89.90± 0.59
Quercetin	95.86± 0.75	85.33± 0.78

Values are expressed as Mean ± SEM of three replicate determinations (n = 3)

Table 2: Effect of *Z. oxyphylla* (100, 250, 500 mg/Kg) on ALT, AST, ALP, albumin, bilirubin and total proteins in paracetamol-induced hepatotoxicity.

Parameters	Control	Positive control	Standard drug control	ZO-100	ZO-250	ZO-500
ALT	39.00±5.52	152.50±49.08	54.50±8.16*	73.17 ±15.76*	58.83 ±3.64*	85.00 ±7.30*
AST	42.50±6.28	194.67±38.53	68.67±18.43**	57.33 ±7.02***	81.33±7.83**	76.50 ±6.16**
ALP	198.33 ±22.49	302.50±28.36	190.67 ±4.59*	197.83±28.08*	205.50±23.51*	244.67±15.4^{ns}
Albumin	3.85 ±0.12	3.12 ±0.12	3.77 ±0.09^{ns}	4.07±0.16$^{\#}$	3.77 ±.09$^{\#}$	3.73 ±0.09$^{\#}$
Bilirubin	0.50 ±0.04	0.75 ±0.07	0.45 ±0.02*	0.60±0.06	0.48 ±0.03**	0.53 ±0.04$^{\#}$
Total Protein	6.47±0.16	5.25 ±0.10	6.08 ±0.15$^{\#}$	6.18±0.17$^{\#}$	6.18 ±0.12$^{\#}$	6.10 ±0.13$^{\#}$

Values are mean ± SEM (n = 6). Where * = P<0.05, ** = P<0.05 significant decrease compared to positive control and # = P>0.05 significant increase compared to positive control
ZO; *Ziziphus oxyphylla,* ALT: aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; *** Values are highly significant statistically at P< 0.001 compared to paracetamol control group.

Table 3: Effect of *Z. oxyphylla* (100, 250, 500 mg/Kg) on blood triglycerides, high-density lipoproteins, low-density lipoproteins in paracetamol-induced hepatotoxicity.

Parameters	Control	Positive control	Standard drug control	ZO-100	ZO-250	ZO-500
TC	64.50±2.36	98.00±6.68	62.50 ±1.6**	74.33±6.4*	71.83 ±7.7*	68.33 ±6.09*
TG	51.00 ±3.24	93.33 ±5.35	57.17 ±2.5**	9.67±4.6**	62.00 ±6.8**	53.33 ±6.68*
HDL	18.50±0.85	12.67 ±0.49	18.67 ±0.7$^{\#}$	18.67±1.8$^{\#}$	20.33 ±1.8$^{\#}$	18.00 ±0.6$^{\#}$
LDL	48.67±4.77	75.50 ±3.35	53.00 ±1.98**	58.00 ±5.3*	52.67 ±3.4*	46.17 ±6.2**

Values are mean ± SEM (n = 6). Where * = P<0.05, ** = P<0.05 significant decrease compared to positive control and $^{\#}$ = P>0.05 significant increase compared to positive control
ZO: *Ziziphus oxyphylla,* TC: total cholesterol; TG:triglycerides; HDL; high density lipoproteins; LDL: low density lipoproteins.

Table 4: Effect of *Z. oxyphylla* (100, 250and 500 mg/Kg) on SOD, GR, MDA and catalase levels in paracetamol-induced hepatotoxicity

Parameters	Control	Positive control	Standard drug control	ZO-100	ZO-250	ZO-500
SOD	106.93±6.37	66.43 ±1.33	112.76±6.78$^{\#}$	101.43 ±3.4$^{\#}$	113.00±11.2$^{\#}$	184.93±17.3$^{\#\#\#}$
GR	203.09 ±8.28	156.55 ±5.55	214.20±13.5$^{\#\#\#}$	191.81±3.9$^{\#}$	207.34±11.7$^{\#\#}$	199.24±7.2$^{\#\#}$
MDA	1.39±0.06	2.37 ±0.19	1.40±0.1***	1.70±0.08$^{\#\#\#}$	1.82±0.06*	1.64±0.11*
CATALASE	5.01±0.17	3.73 ±0.06	4.29±0.11$^{\#}$	4.61±0.13$^{\#}$	5.10±0.06$^{\#}$	4.94±0.08$^{\#}$

Values are mean ± SEM (n = 6). Where * = P< 0.05, ** = P< 0.05 significant decrease compared to positive control, $^{\#}$ = P>0.05, $^{\#\#}$= P >0.01 and $^{\#\#\#}$= P>0.01significant increase compared to positive control
ZO: *Ziziphus oxyphylla,* SOD: Superoxide dismutase; GR: Glutathione reductase; MDA: Malondialdehyde

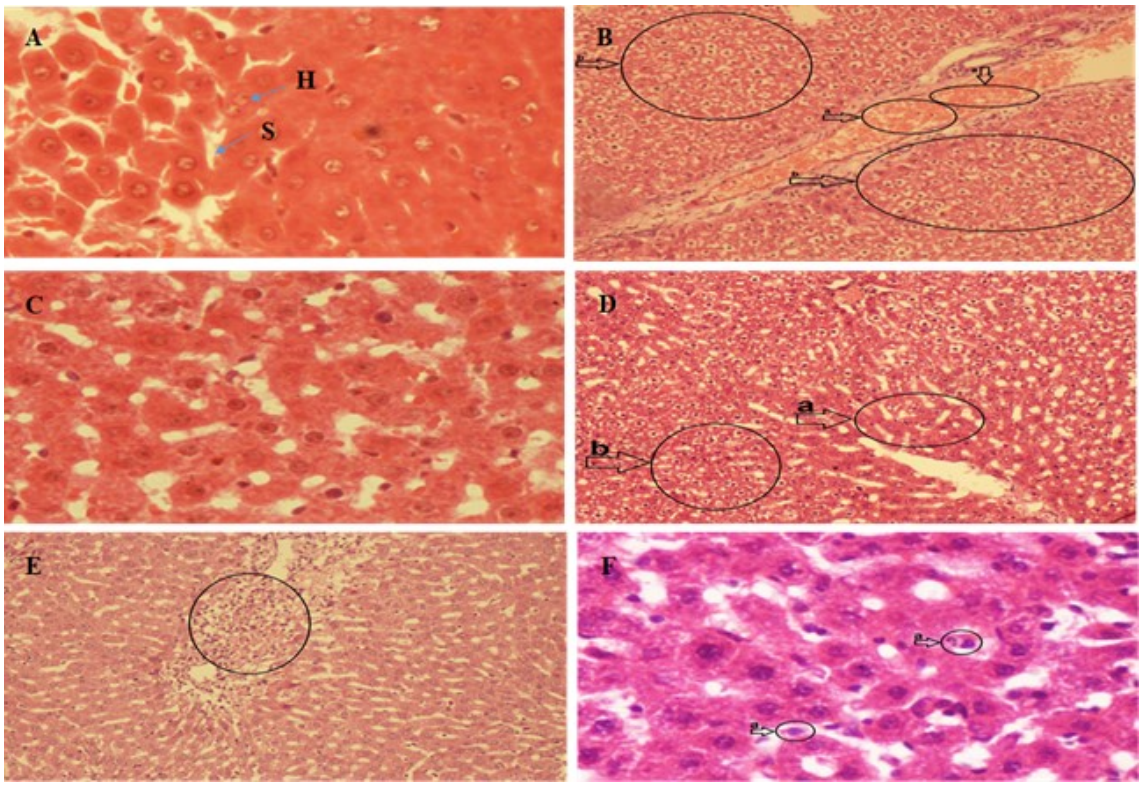

Fig. 1: Histopathological study of livers from control, paracetamol-treated, standard drug control and *Z. oxyphylla* (100, 250 and 500 mg/Kg) treated groups of rats. (A) control group; normal hepatocytes are evident. Sinusoidal spaces are normal. (H; hepatocytes. S; sinusoids) (B) paracetamol treated group; area "a" indicating haemorrhage, area "b" showing congestion of blood vessels and severe hydropic degeneration (C) standard drug control group; recovery of normal hepatocytes with normal sinusoidal spaces. (D)*Z. oxyphylla* (100 mg/Kg) treated group; Area "a" is showing vacuole formation indicating necrosis. Area "B" indicating small hydropic degeneration/Vacuolation. (E) *Z. oxyphylla* (250 mg/Kg) treated group; monocytes and lymphocytes are predominant in the area. (F) *Z. oxyphylla* (500 mg/Kg) treated group; area "a" normal hepatocytes. In other area hepatocyte is turning to be normal. (H & E stain, 400 X).

Effect of Z. oxyphylla on liver biomarkers in paracetamol-induced hepatotoxicity

Our findings showed an increase in ALT, AST and ALP levels in paracetamol treated group of rats as compared to control group. However, treatment with methanol extract of *Z. oxyphylla* (100, 250 and 500 mg/Kg) significantly decreased paracetamol-induced rise in serum enzymes. Results were comparable with those of standard drug as shown in table 2. Paracetamol-induced hepatotoxicity model showed reduction in plasma proteins levels in disease control group, while methanol extract increased the plasma proteins levels.

Effect of Ziziphus oxyphylla on blood lipid profile

An increase in plasma cholesterol, TG and LDL with decrease in HDL level as compared to control group was observed in paracetamol-induced hepatotoxic model. However, treatment with methanol extract of *Z. oxyphylla* (100, 250 and 500 mg/Kg) significantly decreased paracetamol-induced rise in TC, TG and LDL and increased HDL levels. Results were comparable with those of standard drug shown in table 3.

Effect of Ziziphus oxyphylla on oxidative stress

Methanol extract of *Z. oxyphylla* showed increase in SOD, GR and catalase levels but reduced MDA level as compared with positive control group as shown in table 4.

Histopathological Study

Histopathological studies demonstrated that control group showed normal lobular architecture and normal hepatic cells. However, livers from paracetamol treated animals showed massive necrosis, hemorrhage and inflammation with lymphocytes infiltration. These pathological changes were reduced with methanol extract of *Z. oxyphylla* indicating its ability to reverse the paracetamol-induced intoxication as shown in figure 1.

DISCUSSION

Liver plays a key role in the metabolic and physiological processes of the body. However, free radicals, alcohol, xenobiotics and pollutants may cause disturbance in normal functioning of the liver (Migliaccio *et al*., 2019).

A number of hepatotoxicity animal models have been used to evaluate the hepatoprotective activity of the plant extracts. Acetaminophen (paracetamol)-induced toxicity in rats is one of the commonly used experimental model (Hussain *et al*., 2014). A large number of phytochemicals extracted from plants have been used for treatment of various diseases nowadays. It becomes necessary to evaluate natural chemicals as effective alternatives to costly and side effects associated drugs. The aim of the present study was to evaluate the hepatoprotective activity of methanolic extract of the aerial parts of *Z. oxyphylla* in paracetamol-induced hepatotoxicity animal model. Liver function tests, blood lipid profile, liver antioxidant enzymes have been assessed to evaluate hepatoprotective activity of the plant. Firstly, free radical scavenging ability of petroleum ether, chloroform and methanol extracts of the aerial parts of *Z. oxyphylla* were investigated. The methanol extract was found to be more effective as shown in table 1; hence further *in vivo* studies were carried out by using methanol extract of *Z. oxyphylla*.

Paracetamol causes liver damage when taken in high doses. It is metabolized to N-acetyl p-benzoquinone imine (NAPQI) that binds covalently to tissue macromolecules, cause oxidation of lipids and sulfhydryl groups and modifies homeostasis of calcium ions (Ramachandran *et al.*, 2019). However, a large production of reactive species cause consumption of protective moieties such as glutathione and α-tocopherol, which may lead to cell membrane damages and hence liver injury (Truong *et al*., 2018). Damage to cell membranes leads to leakage of cytoplasmic ALT, AST, ALP, bilirubin, total cholesterol, total protein and albumin into serum (MCGill, 2016). In the present study, a rise in serum levels of ALT, AST, ALP, bilirubin, and cholesterol as well as decrease in total protein and albumin levels have been found in positive control group (paracetamol-treated) showing the damage to liver cells by paracetamol (table 2). Pre-treatment with *Z. oxyphylla* restored the serum levels of enzymes in hepatotoxicity model. It might be due to membrane stabilizing effect of the *Z. oxyphylla* extract that prevented the leakage of enzymes. Our findings agree with the previous study (Jena *et al*., 2019). Destruction of liver cells by paracetamol caused reduction in serum albumin and total proteins in this study. The reverse effect has been achieved by pre-treatment with *Z. oxyphylla* extract indicating an improvement of the liver cells functioning.

In the present study, paracetamol treatment increased TC, TG, LDL and decreased HDL levels. It has been reported earlier that paracetamol-induce deterioration of liver cells which may lead decreased ability of cells to metabolize lipids (Lambers *et al*., 2018). Pre-treatment with the methanol extract of *Z. oxyphylla* caused significant reduction of TC, TG, LDL and increase in HDL levels in paracetamol treated group as shown in table 3. *Z. oxyphylla* might show protective activity against the deleterious effects of paracetamol on hepatic cells.

Antioxidant enzymes such as SOD, catalase and GR play an important role in protecting the tissues from reactive oxygen species (Abirami *et al*., 2015). Reduced levels of these enzymes in positive control group indicated the lipid peroxidation with paracetamol treatment in this study as shown in table 4. Pre-treatment with *Z. oxyphylla* (100, 250 and 500 mg/Kg) dose dependently increased SOD, catalase and GR levels. This effect might be due to the ability of *Z. oxyphylla* to reduce oxidative damage to hepatic cells by paracetamol while increasing the level of MDA in methanol extract treated rats. Moreover, histopathological studies also confirmed our findings. Control group showed normal lobular architecture and normal hepatic cells. Necrosis, haemorrhage and inflammation with lymphocytes infiltration were observed in the liver cells of paracetamol treated rats. These pathological changes were reduced with methanol extract of *Z. oxyphylla* indicating its ability to reverse the paracetamol-induced intoxication. Previous studies have shown that flavonoids (glycosides of quercetin and kaempferol) are responsible for the anti-oxidant activity of *Z. oxyphylla*. The membrane stabilizing effect and the decreased in paracetamol induced oxidative stress by *Z. oxyphlla* might involve these flavonoid glycosides. These flavonoids have shown well defined anti-oxidant potential (Ahmad *et al*., 2016).

CONCLUSIONS

It is concluded that the methanol extract of *Z. oxyphylla* possesses hepatoprotective activity that might be due to quercetin and kaempferol glycosides present in plant extract. Further studies are required to elucidate the exact mechanism of action of these isolated glycosides.

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